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(54) HEMATOPOIETIC ORGAN TUMOR CELL DETECTION METHOD AND HEMATOPOIETIC ORGAN TUMOR CELL DETECTION KIT

(57) Abstract:

PROBLEM TO BE SOLVED: To provide a hematopoietic organ tumor cell detection method which detects with high sensitivity and high specificity a hematopoietic organ tumor cell, and a detection kit.

SOLUTION: The hematopoietic organ tumor cell detection method comprises the steps of: quantifying a tyrosine phosphatase SHP1 protein specific to a hematopoietic organ cell included in a specimen sample containing the hematopoietic organ cell; and identifying methylation of CpG islands included in the base sequencing of the SHP1 gene obtained from the abovementioned specimen sample. Thus, since the presence or absence of the hematopoietic organ tumor cell is identified at two stages by one genetic information, the hematopoietic organ tumor cell is detectable by a very high specificity.

CLAIMS

[Claim(s)]

[Claim 1]

- (1) A SHP1 gene-methylation check process which checks methylation of a CpG island included in a base sequence of protein tyrosine phosphatase SHP1 gene specific into a hematopoietic organ cell included in a sample sample containing a hematopoietic organ cell,
- (2) a SHP1 gene-product fixed-quantity process of quantifying at least one expression amount of SHP1 protein obtained from the above-mentioned sample sample, and SHP1mRNA -- and
- (3) A SHP1 gene LOH check process which checks existence of heterozygosity loss (LOH) of SHP1 gene contained in the above-mentioned sample sample,
- ** -- a hematopoietic organ tumor cell detection method by which it being included any they are even if small.

[Claim 2]

In the above-mentioned SHP1 gene-methylation check process

A gene cutting trial stage processed with a methylation sensitivity restriction enzyme which recognizes a base sequence which includes a genetic material obtained from the abovementioned sample sample for cytosine,

A gene amplification trial stage of enforcing a polymerase chain reaction method (PCR) using a primer which amplifies a field which is included in a base sequence of the SHP1 abovementioned gene, and includes a base sequence by which recognition cutting is carried out in the above-mentioned methylation sensitivity restriction enzyme to a gene processed with the above-mentioned methylation sensitivity restriction enzyme,

The hematopoietic organ tumor cell detection method according to claim 1, wherein the amount check stage of gene amplification of checking quantity of a gene of amplified specific size is included.

[Claim 3]

The hematopoietic organ tumor cell detection method according to claim 2, wherein the above-mentioned primer is a partial base sequence further included in a base sequence shown in the array number 1 or 2, or the polynucleotide which has this partial base sequence and complementarity.

[Claim 4]

The hematopoietic organ tumor cell detection method according to claim 2 or 3 characterized by checking quantity of a gene of specific size using an electrophoresis method in the abovementioned amount check stage of gene amplification.

[Claim 5]

The hematopoietic organ tumor cell detection method according to claim 2, 3, or 4 characterized by using a methylation sensitivity restriction enzyme as a restriction enzyme in the abovementioned gene cutting trial stage.

[Claim 6]

In the above-mentioned SHP1 gene-methylation check process

A gene ornamentation stage of processing a genetic material obtained from the above-mentioned sample sample by a bisulfite,

The hematopoietic organ tumor cell detection method according to claim 1, wherein a methylation cytosine content judging stage included in a genetic material processed by a bisulfite

of judging existence of methylation cytosine in a base sequence of SHP1 gene is included. [Claim 7]

A way PCR detects methylation cytosine in the above-mentioned methylation cytosine content judging stage, The hematopoietic organ tumor cell detection method according to claim 6, wherein it is used at least among a way determination of a base sequence of a gene detects methylation cytosine, or a method of identifying a base sequence containing methylation cytosine any they are.

[Claim 8]

The hematopoietic organ tumor cell detection method according to claim 6 or 7 characterized by using sodium bisulfite as a bisulfite in the above-mentioned gene ornamentation stage. [Claim 9]

A hematopoietic organ tumor cell detection method given in any 1 paragraph of claims 1 thru/or 8 quantifying SHP1 protein in the above-mentioned SHP1 gene-product fixed-quantity process using SHP1 antibody which uses SHP1 protein as an antigen.

[Claim 10]

The hematopoietic organ tumor cell detection method according to claim 9 characterized by quantifying SHP1 protein by enzyme-labeled antibody technique or a western blotting method in the above-mentioned SHP1 gene-product fixed-quantity process.

[Claim 11]

By detecting a manifestation of mRNA of SHP1 gene using polynucleotide which detects an overall length of a base sequence, or its part of SHP1 gene cDNA shown in the array number 3 in the above-mentioned SHP1 gene-product fixed-quantity process, A hematopoietic organ tumor cell detection method given in any 1 paragraph of claims 1 thru/or 8 quantifying SHP1mRNA. [Claim 12]

In the above-mentioned SHP1 gene-product fixed-quantity process, a northern blotting method, The reverse transcription PCR method, the real-time PCR method, or RNA in The hematopoietic organ tumor cell detection method according to claim 11, wherein a manifestation of mRNA of SHP1 gene is detected by a situ hybridization method.

[Claim 13]

Two Microsatellite markers which put the SHP1 above-mentioned gene at least a check of existence of heterozygosity loss on the other hand, Or a hematopoietic organ tumor cell detection method given in any 1 paragraph of claims 1 thru/or 12 carrying out gene polymorphism like simple nucleotide polymorphism which exists the inside of the above-mentioned SHP gene, and near it in the fragmentation analysis using PCR.

[Claim 14]

It is used in order to detect hematopoietic organ tumor cells from a sample sample containing a hematopoietic organ cell,

- (1) SHP1 antibody which uses protein tyrosine phosphatase SHP1 protein specific into a hematopoietic organ cell as an antigen -- and
- (2) A methylation sensitivity restriction enzyme which recognizes a base sequence containing cytosine,

A hematopoietic organ tumor cell detection kit comprising:

A primer for PCR which amplifies a field which is included in a base sequence of SHP1 gene and includes a base sequence recognized by the above-mentioned methylation sensitivity restriction enzyme.

the methylation positivity of the SHP1 above-mentioned gene, and inside with methylation

negative control DNA -- at least -- on the other hand.

[Claim 15]

It is used in order to detect hematopoietic organ tumor cells from a sample sample containing a hematopoietic organ cell,

- (1) SHP1 antibody which uses protein tyrosine phosphatase SHP1 protein specific into a hematopoietic organ cell as an antigen,
- (2) a primer for a judgment of existence of cytosine in a base sequence of SHP1 gene contained in a genetic material processed by bisulfite refined to a gene processing level, and this bisulfite -- and
- (3) A hematopoietic organ tumor cell detection kit containing any at least one of primers for PCR which detect an overall length of a base sequence, or its part of SHP1 gene cDNA shown in the array number 3.

[Claim 16]

It is used in order to detect hematopoietic organ tumor cells from a sample sample containing a hematopoietic organ cell,

A hematopoietic organ tumor cell detection kit containing a primer for PCR which detects at least one overall length of two Microsatellite markers which put protein tyrosine phosphatase SHP1 specific gene between a hematopoietic organ cell, or its part.

[Claim 17]

The hematopoietic organ tumor cell detection kit according to claim 14, 15, or 16 containing either [at least] a reagent for PCR, or a reagent for a restriction enzyme reaction.

DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

This invention relates to the hematopoietic organ tumor cell detection kit used suitably for a hematopoietic organ tumor cell detection method and this detecting method.

By detecting methylation of SHP1 gene which encodes manifestation reduction, disappearance, or this of protein tyrosine phosphatase SHP1 specific gene product to a malignant lymphoma, leukemia, etc. especially, for example, It is related with the detecting method and detection kit which can detect hematopoietic organ tumor cells on high sensitivity and a high unique target.

[0002]

[Description of the Prior Art]

Various kinds from what has a very bad prognosis to what has a comparatively good prognosis are known for intractableness by hematopoietic organ tumors (tumor of blood systems) in Homo sapiens (Homo sapiens), such as a malignant lymphoma and leukemia. Although various therapies, such as various chemotherapies, radiotherapy, or immunotherapy, are already put in practical use by the therapy of this hematopoietic organ tumor, even if tumor cells carry out regression mostly as a result of such a therapy, if tumor cells survive slightly, the relapse of a hematopoietic organ tumor will not be escaped.

[0003]

Diagnosis of the above-mentioned hematopoietic organ tumor is synthetically carried out by using two or more modalities together by the former. Using peripheral blood and various biopsy specimens, the morphological observation and the histological observation by a tissue staining color, immunity dyeing, etc. are carried out, or, specifically, further various molecular biological analyses, chromosome analyses, etc. are carried out. Diagnosis of the above-mentioned hematopoietic organ tumor takes time most by judgment. [0004]

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention]

each above-mentioned conventional modality -- it -- if independent -- hematopoietic organ tumor cells -- high sensitivity -- high -- it is not specifically and promptly detectable. So, in the modality of these former, if plurality is used together and it does not judge synthetically, a hematopoietic organ tumor cannot be diagnosed.

[0005]

That is, as long as the conventional modality is used, in order to have to use two or more modalities together, it not only causes complicated-ization of diagnosis, but time is taken, and since neither hematopoietic organ tumor cell detection sensitivity nor singularity is high, a medical practitioner's special judgment will occupy big specific gravity to diagnosis. Therefore, in the former, the diagnostic technique of a hematopoietic organ tumor is not used for the purpose of the early detection and early treatment of the hematopoietic organ tumor by a mass screening, although it is substantially restricted to use in a medical site and can respond to each disease person.

[0006]

In order to carry out hematopoietic organ tumor cells on high sensitivity and a high unique target more, it is possible to use the marker with high sensitivity specifically looked at by the hematopoietic organ tumor of the wide range to hematopoietic organ tumor cells. If such a marker is used, early detection and diagnosis of a hematopoietic organ tumor can be carried out easily and promptly, It not only becomes possible to apply to the early treatment and recurrence prevention of a malignant lymphoma, leukemia, etc., but on medical science, it can consider it as available diagnostic technique at clinical laboratory test industry, pharmaceutical industry, etc., and it becomes possible to contribute to development of industry. However, such a marker is not known the place by the present.

[0007]

This invention is made in view of an aforementioned problem, and the purpose, Using molecular biological knowledge, hematopoietic organ tumor cells are detected from a small amount of patient samples on high sensitivity and a high unique target promptly and simple, the early detection, diagnosis, and early treatment of a hematopoietic organ tumor are made easy, and it is in providing a hematopoietic organ tumor cell detection method and a detection kit applicable also to a mass screening.

[8000]

MEANS

[Means for Solving the Problem]

As a result of inquiring wholeheartedly in view of an aforementioned problem, this invention persons in a large malignant hematopoietic organ tumor of a range. In a hematopoietic organ tumor with malignancy high moreover in which expression inhibition of protein tyrosine phosphatase SHP1 protein is extremely seen by high frequency, By using as a marker both sides of SHP1 gene which finds out that a tendency of expression inhibition of the SHP1 abovementioned protein becomes strong, and encodes SHP1 gene product and this. high sensitivity of hematopoietic organ tumor cells -- high -- a thing [specific and] for which hematopoietic organ tumor cell detection art which can be detected in a short time and can be used industrially can be realized is found out, and it came to complete this invention.

Namely, a hematopoietic organ tumor cell detection method concerning this invention, . In order to solve the above-mentioned technical problem, are contained in a sample sample containing (1) hematopoietic organ cell. A SHP1 gene-product fixed-quantity process of quantifying at least one expression amount of protein tyrosine phosphatase SHP1 specific protein and SHP1mRNA into a hematopoietic organ cell, (2) A SHP1 gene-methylation check process which checks methylation of a CpG island included in a base sequence of SHP1 gene acquired from the above-mentioned sample sample, And it is characterized by including at least one side of SHP1 gene LOH check process ** which checks existence of heterozygosity loss (LOH) of SHP1 gene contained in the (3) above-mentioned sample sample.

This phenomenon is not looked at by normal blood cell to expression inhibition of the SHP1 above-mentioned gene product being extremely looked at by malignant hematopoietic organ tumor cells by high frequency. Expression inhibition of the SHP1 above-mentioned protein is based on methylation of SHP1 gene. One allele of SHP1 gene has lost before and after transfer control of SHP1 gene by DNA methylation.

By according to the described method, checking methylation of SHP1 gene obtained from a sample sample using the above-mentioned knowledge, and detecting existence of hematopoietic organ tumor cells. screening existence of existence of malignant hematopoietic organ tumor cells -- on the other hand -- SHP in a sample sample -- a manifestation of SHP1 protein, SHP1mRNA, or its both is specifically quantified 1 gene product. [0012]

That is, in a described method, inactivation of SHP1 gene can be judged using four-fold marker by the maximum called loss of ornamentation and mRNA of gene DNA, protein, and allele. namely, one hematopoietic organ tumor cell called a SHP1 gene-expression fall -- since a specific phenomenon can be checked in four steps, hematopoietic organ tumor cells are detectable by very high singularity.

[0013]

As a desirable example of a hematopoietic organ tumor cell detection method concerning this invention, A gene cutting trial stage processed with a methylation sensitivity restriction enzyme which recognizes a base sequence which includes in the above-mentioned SHP1 genemethylation check process a genetic material obtained from the above-mentioned sample sample for cytosine, It is contained in a base sequence of the SHP1 above-mentioned gene to a gene processed with the above-mentioned methylation sensitivity restriction enzyme, A detecting method with which a gene amplification trial stage of enforcing the PCR method using a primer

which amplifies a field including a base sequence by which recognition cutting is carried out, and the amount check stage of gene amplification of checking quantity of a gene of amplified specific size are included in the above-mentioned methylation sensitivity restriction enzyme can be mentioned.

[0014]

According to the described method, after distinguishing existence of methylation by trying cutting of SHP1 gene contained in a genetic material obtained from a sample sample using a methylation sensitivity restriction enzyme and amplifying using PCR further, quantity of an PCR product of specific size obtained is checked. So, if even a little SHP1 genes are obtained from a sample sample, methylation of SHP1 gene is detectable. Therefore, even if hematopoietic organ tumor cells exist very much in a sample sample only in a minute amount, it is high detection sensitivity, and it becomes possible to detect hematopoietic organ tumor cells on a high unique target moreover.

[0015]

In the above-mentioned detecting method, it is preferred that the above-mentioned primer is a partial base sequence further included in a base sequence shown in the array number 1 or 2 or the polynucleotide which has this partial base sequence and complementarity.

[0016]

In the above-mentioned detecting method, it is preferred to check quantity of a gene of specific size in the above-mentioned amount check stage of gene amplification using an electrophoresis method.

[0017]

In the above-mentioned detecting method, it is preferred to use a restriction enzyme in which a methylation insusceptible restriction enzyme which recognizes the same base sequence is known as a methylation sensitivity restriction enzyme in the above-mentioned gene cutting trial stage. [0018]

As other examples with a preferred hematopoietic organ tumor cell detection method concerning this invention, A gene ornamentation stage of processing a genetic material obtained from the above-mentioned sample sample by a bisulfite to the above-mentioned SHP1 gene-methylation check process, A detecting method with which a methylation cytosine content judging stage included in a genetic material processed by a bisulfite of judging existence of methylation cytosine in a base sequence of SHP1 gene is included can be mentioned.

If a genetic material obtained from a sample sample using a bisulfite is processed according to the described method, cytosine in a base sequence will be changed into uracil, but methylated cytosine is not changed. Therefore, methylation of SHP1 gene is detectable only by judging whether cytosine is contained in a base sequence of SHP1 gene after a gene ornamentation stage. Therefore, it becomes possible to detect hematopoietic organ tumor cells on a high unique target by a simple mechanism.

[0020]

In the above-mentioned detecting method, in the above-mentioned methylation cytosine content judging stage. It is desirable even if it is used at least among processings of a gene by way PCR detects methylation cytosine, way determination of a base sequence of a gene detects methylation cytosine, or a method of identifying a base sequence containing methylation cytosine any they are.

[0021]

By using PCR at least according to the described method, if even a little SHP1 genes are obtained from a sample sample, methylation of SHP1 gene is detectable. Therefore, even if hematopoietic organ tumor cells exist very much in a sample sample only in a minute amount, it becomes possible to detect hematopoietic organ tumor cells on a high unique target by high detection sensitivity.

[0022]

In the above-mentioned detecting method, it is preferred that sodium bisulfite is used as a bisulfite in the above-mentioned gene ornamentation stage. In the above-mentioned gene ornamentation stage, urea may be used together with a bisulfite.

In a hematopoietic organ tumor cell detection method concerning this invention, even if it is a detecting method of which example of the above, when SHP1 protein is quantified in the above-mentioned SHP1 gene-product fixed-quantity process using SHP1 antibody which uses SHP1 protein as an antigen, it is desirable. Specifically in the above-mentioned SHP1 gene-product fixed-quantity process. It is desirable when SHP1 protein is quantified by enzyme-labeled antibody technique (an immunohistochemical method, an immunocytochemistry method, the ELISA (enzyme-linked immunosorbent assay) method) or a western blotting method. 100241

Since SHP1 protein will be quantified using an antigen-antibody reaction according to the described method, it becomes possible to detect hematopoietic organ tumor cells on a high unique target by a simple mechanism.

[0025]

Or in a hematopoietic organ tumor cell detection method concerning this invention, Even if it is a detecting method of which example of the above, in the above-mentioned SHP1 gene-product fixed-quantity process. By detecting a manifestation of mRNA of SHP1 gene using polynucleotide which detects an overall length of a base sequence, or its part of SHP1 gene cDNA shown in the array number 3, even if it quantifies SHP1mRNA, it is desirable. Specifically at the above-mentioned SHP1 gene-product fixed-quantity process, they are a northern blotting method, the reverse transcription PCR method, the real-time reverse transcription PCR method, a situ hybridization method.

[0026]

Since SHP1 gene product will be quantified by mRNA of SHP1 gene as SHP1 gene product according to the described method, a mechanism simple by using oligopeptide which has cDNA and homology of SHP1 gene as a probe or a primer -- high -- it becomes possible specific and to detect hematopoietic organ tumor cells to high sensitivity.

[0027]

a hematopoietic organ tumor cell detection method concerning this invention -- it is desirable -- as other examples to a pan, Two Microsatellite markers which put the SHP1 above-mentioned gene at least a check of existence of heterozygosity loss on the other hand, Or a method enforced by [which used PCR] conducting fragmentation analysis in gene polymorphism like simple nucleotide polymorphism which exists the inside of the above-mentioned SHP1 gene and near it can be mentioned. The sample sample used at this time should just be a sample sample containing a hematopoietic organ cell. As contrast, a sample obtained after complete hematological remission may be used, and other normal tissue cells may be used. [0028]

Since heterozygosity loss of SHP1 gene is checked by checking heterozygosity loss of gene polymorphism, such as a Microsatellite marker or simple nucleotide polymorphism (SNP), by PCR according to an above-mentioned method, It becomes possible to detect hematopoietic organ tumor cells more certainly by a simple mechanism. [0029]

As a desirable example of a hematopoietic organ tumor cell detection kit concerning this invention, It is used in order to detect hematopoietic organ tumor cells from a sample sample containing a hematopoietic organ cell, (1) A methylation sensitivity restriction enzyme which recognizes a base sequence which contains in a hematopoietic organ cell SHP1 antibody which uses protein tyrosine phosphatase SHP1 specific protein as an antigen, and (2) cytosine, Composition containing at least one side can be mentioned among a primer for PCR which amplifies a field which is included in a base sequence of SHP1 gene and includes a base sequence by which recognition cutting is carried out in the above-mentioned methylation sensitivity restriction enzyme, and the methylation positivity of the SHP1 above-mentioned gene and methylation negative control DNA.

[0030]

Or as other examples with a preferred hematopoietic organ tumor cell detection kit concerning this invention, A bisulfite which was used in order to detect hematopoietic organ tumor cells from a sample sample containing a hematopoietic organ cell, and was refined to (1) abovementioned SHP1 antibody and (2) gene processing level, A primer for a judgment of existence of cytosine in a base sequence of SHP1 gene contained in a genetic material processed by this bisulfite, And composition containing any at least one of primers with an overall length, or its part and homology of a base sequence of the SHP1 gene cDNA shown in the (3) array number 3 for PCR can be mentioned.

[0031]

As other examples in which a hematopoietic organ tumor cell detection kit concerning this invention is preferred, It is used in order to detect hematopoietic organ tumor cells from a sample sample containing a hematopoietic organ cell, Composition containing a primer for PCR which detects at least one overall length of two Microsatellite markers which put protein tyrosine phosphatase SHP1 specific gene between a hematopoietic organ cell, or its part can be mentioned.

[0032]

In the above-mentioned hematopoietic organ tumor cell detection kit, it is still more preferred that either [at least] a reagent for PCR or a reagent for a restriction enzyme reaction is included. [0033]

Even if it is which composition of the above, in order to enforce a hematopoietic organ tumor cell detection method mentioned above, desirable drugs, a specimen, etc. are contained. Therefore, a hematopoietic organ tumor cell detection method concerning this invention can be enforced easily and promptly by using the above-mentioned detection kit, and it becomes possible to use this invention on industrial levels, such as clinical laboratory test industry and pharmaceutical industry.

[0034]

[Embodiment of the Invention]

[Embodiment 1]

It will be as follows if one gestalt of the operation in this invention is explained based on drawing 1 thru/or drawing 24. This invention is not limited to this.

[0035]

While this invention quantifies protein tyrosine phosphatase SHP1 gene product, i.e., SHP1 protein, and mRNA from a promotor specific into a hematopoietic organ cell which are contained in the sample sample containing a hematopoietic organ cell, It is the art of detecting hematopoietic organ tumor cells out of the above-mentioned sample sample by checking the methylation of the CpG island included in the base sequence of SHP1 gene acquired from the above-mentioned sample sample. [0036]

SHP1 gene used as a marker for detecting hematopoietic organ tumor cells by this invention, Exist in the chromosome 12p13 and the base sequence shown in <u>drawing 1 - drawing 10</u>, and the array number 1 is made into the sense strand of genomic DNA (wild type), It is a gene which has an exon (a figure and an array table Chuo University field shown in written form) of 16 which makes an antisense strand the base sequence shown in <u>drawing 11 - drawing 20</u>, and the array number 2. The cDNA has the size of about 1.8 kbs which have a base sequence shown in <u>drawing 21</u> and the array number 3. SHP1 gene is the same gene as SH-PTP1, PTP1C, HCP, HCPH, PTPN6, HPTP1C, and SHP-1L.

[0037]

SHP1 protein by which the code is carried out to the SHP1 above-mentioned gene, As it is protein tyrosine phosphatase (PTPase) specific into various hematopoietic organ cells and is shown in <u>drawing 22</u> by molecular weight 68kD, It has the structure of having two SH2 (Src homology domain 2) fields (270 amino acid residue) which serve as tandem construction at the amino terminal side, a PTPase domain of 246 amino acid residue, and the C terminal side field of 93 amino acid residue. It has an amino acid sequence shown in <u>drawing 23</u> and the array number 4.

[0038]

In a human hematopoietic organ tumor, for example, a malignant lymphoma, and leukemia. The strong expression inhibition of SHP1 protein is seen by not less than 90% of high frequency by many kinds (for example, refer to American Journal of Pathology, Vol.159, No.4, and October2001:1495-1505 grade). Thus, in malignant hematopoietic organ tumor cells, this phenomenon is not looked at by the normal blood cell to the expression inhibition of the SHP1 above-mentioned protein being extremely seen by high frequency. [0039]

This invention persons found out uniquely that the expression inhibition of the SHP1 above-mentioned protein made a cause the transfer abnormality by the SHP1 above-mentioned gene being methylated.

[0040]

For example, as shown in <u>drawing 24</u>, in the sense strand (it illustrates up to 181 bases - 2160 bases) of the genomic DNA (wild type) shown in <u>drawing 1</u> - <u>drawing 10</u>, and the array number 1, promoterregion exists before the exon (figure Chuo University character) of 1001 bases - 1163 bases, but. CG arrangement with which cytosine (C) and guanine (G) are located in a line exists in this neighborhood mostly, and forms the CpG island (CpG island) in it (in <u>drawing 24</u>, shading of a bold letter shows CG arrangement). Although cytosine of this CpG island is not methylated in a normal hematopoietic organ cell, many of cytosine of the above-mentioned CG arrangement is methylated, for example by the malignant cell lymphoma. Of course, methylation of cytosine in this CG arrangement is produced not only like a sense strand but like an antisense strand.

[0041]

Advanced methylation of CG arrangement in the above-mentioned CpG island prevents transfer of mRNA from DNA of SHP1 gene, and, as a result, production of SHP1 protein is controlled. It is extremely concluded by high frequency by hematopoietic organ tumor cells that this phenomenon was mentioned above. And methylation of DNA in SHP1 gene disappears thoroughly, and very high correlation is seen between the knowledge on molecular biology, and the knowledge on clinical at various hematopoietic organ tumor patients' complete remission term. So, it is guessed that the SHP1 gene-expression control by methylation has played the important role in the onset mechanism of hematopoietic organ tumor cells. So, in this invention, the phenomenon of the above-mentioned SHP1 gene-expression control is used as a marker of hematopoietic organ tumor cells.

[0042]

It also found this invention persons uniquely that one allele of SHP1 gene loses before and after transfer control of SHP1 gene arises according to the DNA methylation mentioned above, when the symptoms of diseases, such as a malignant lymphoma and leukemia, develop. Then, it becomes possible by checking heterozygosity loss of SHP1 gene to check loss of the allele of SHP1 gene. So, heterozygosity loss of SHP1 gene can also be used as a marker of hematopoietic organ tumor cells.

[0043]

In a malignant lymphoma or leukemia, a fall or disappearance of DNA methylation of high frequency, heterozygosity loss of high frequency, and SHP1 gene expression is detected by SHP1 gene, and it is in the tendency for outpatient department SHP1 transgenics to control growth of the cell of a corpuscle system, further at it. Thereby, it is suggested strongly that SHP1 gene is one of the antioncogenes.

[0044]

Then, methylation of the CpG island included according to a SHP1 gene-methylation check process in this invention in the base sequence of SHP1 gene obtained from the above-mentioned sample sample is checked, Either [at least] SHP1 protein contained in the sample sample which contains a hematopoietic organ cell at a SHP1 gene-product fixed-quantity process, or mRNA is quantified, and three processes of checking heterozygosity loss of SHP1 gene are further used by a SHP1 gene LOH check process. These processes may be used independently and both sides may be used. In a SHP1 gene-product fixed-quantity process, only SHP1 protein may be quantified, only SHP1 mRNA may be detected and both sides may be detected. [0045]

By this, it screens by detecting methylation of SHP1 gene in a sample sample first, for example, Then, the detection process of becoming final and conclusive existence of hematopoietic organ tumor cells can be carried out by checking the existence of malignant hematopoietic organ tumor cells by quantifying the manifestation of SHP1 gene product of a sample sample at least by one side of SHP1mRNA and SHP1 protein.

[0046]

Therefore, in this invention, SHP1 gene expression can be judged using four-fold marker by the maximum called loss of ornamentation and mRNA of gene DNA, protein, and allele. namely, one hematopoietic organ tumor cell called a SHP1 gene-expression fall -- since a specific phenomenon can be checked by a three-stage, hematopoietic organ tumor cells are detectable by very high singularity.

[0047]

As mentioned above, it is also checked that it is in the tendency which controls growth of the cell of a corpuscle system by introducing SHP1 gene in this invention. So, SHP1 gene is possible also for using for gene therapy, for example, can expect to control growth of tumor cells by transfecting tumor cells in a SHP1 gene-expression vector.

[0048]

The sample sample used by this invention is not especially limited no matter it may be what sample sample, if it is a sample sample containing hematopoietic organ cells, such as peripheral blood or bone marrow fluid. With the hematopoietic organ cell in this invention, although various blood cells are included, various leucocytes are mentioned especially preferably. More specifically, a lymphocyte (a T cell and a B cell), granulocyte (neutrophil leucocyte, eosinophile leucocyte, basophilic leucocyte), monocyte and a macrophage, a mast cell, a spontaneous killer cell, etc. can be mentioned. Or they may be a hematopoietic stem cell and lymphoid precursors. [0049]

Therefore, in the sample sample used by this invention. It is good also as a sample sample for analysis which is easy to carry out molecular biology analysis by performing publicly known processing conventionally to the blood and the body fluid which extracted blood, bone marrow fluid or body fluid etc. in which the above-mentioned hematopoietic organ cell is contained from Homo sapiens, could use this as a sample sample as it was, and were extracted. [0050]

As a hematopoietic organ tumor which can apply this invention, specifically, for example, chronic myelogenous leukemia and a Philadelphia chromosome positive (+ (9; 22) (qq34;q11).) Various myeloproliferative disorder, such as BCR/ABL chronic myelogenous leukemia, chronic neutrophil leucocyte leukemia, chronic eosinophile leucocyte leukemia / hypereosinophilic syndrome, chronic outbreak nature bone marrow fibrosis, polycythemia vera, essential thrombocytosis, and other myeloproliferative disorder that cannot be classified; Bone marrow atypicality / myeloproliferative disorder, such as chronic myelogenous monocytic leukemia, atypical chronic-myelogenous-leukemia, and infancy nature myelogenous monocytic leukemia;

Bone marrow atypical syndromes, such as refractory anemia (bone marrow atypical syndrome) accompanied by the intractable hypocytosis (bone marrow atypical syndrome) and superfluous blast cell 5q-syndrome accompanied by the refractory anemia accompanied by ringed sideroblast, refractory anemia without ringed sideroblast, and a multi-series metaplasia, and other bone marrow atypical syndromes which cannot be classified;

The acute myelogenous leukemia (AML) accompanied by a recurrent cytogenetic translocation. for example, AML and AML1(CBF-alpha)/accompanied by + (8; 21) (q22;q22), [ETO and] the myelogenous leukemia before acuteness (AML accompanied by + (15; 17) (q22;q11-12) -- and - the --) [and] PML/RAR-alpha and unusual marrow eosinophil (inv (16), (p13q22), or + (16; 16) (p13;q11).) AML accompanied by CBF beta/MYH 11X, AML accompanied by 11q23 (MLL) abnormalities, AML accompanied by a multi-series metaplasia with front bone marrow atypical syndrome, AML without a multi-series metaplasia with front bone marrow atypical syndrome, AML related to a therapy, and bone marrow atypical syndrome (related to an alkylating agent --) [and] The therapy related to epipodophyllotoxin, or the therapy of other types, AML (a low differentiation type, a thing without maturation, and the thing accompanied by maturation.) which otherwise does not belong to a section acute myelogenous monocytic leukemia, acute monocytic leukemia, acute erythroblast leukemia, acute megakaryocyte leukemia, the acute basophilocytic leukemia, the acute over***** vegetation accompanied by

the bone marrow fibrosis, and acute 2 character -- acute-myelogenous-leukemia [, such as sex leukemia] (AML);

a precursor B cell nature tumor (the precursor B-lymphoblastic leukemia / lymphoma (precursor B cell acute lymphoblastic leukemia).) a mature (tip) B cell nature tumor (B cell chronic lymphocytic leukemia / small lymphocytic lymphoma.) B cell prolymphocytic leukemia, a lymphoplasmacytic lymphoma, spleen verge field B cell lymphoma (+/-villus lymphocyte), Pilliform cell leukemia, plasma cell myeloma (plasmocytoma), the verge type B cell lymphoma outside a MALT type paragraph, **** verge type B cell lymphoma (+/- monocyte type B cell), a follicular lymphoma, a mantle cell lymphoma, diffusion large-sized B cell lymphoma (mediastinum large cell B cell lymphoma, primary exudation lymphoma), and Burkitt B cell nature tumors, such as a lymphoma / Burkitt cell leukemia;

a precursor T cell nature tumor (the precursor T-lymphoblastic leukemia / lymphoma (precursor T cell acute lymphoblastic leukemia).) a mature (tip) T cell nature tumor (T cell prolymphocytic leukemia and T cell granulation lymphocyte leukemia.) Invaded type spontaneous killer cell leukemia, adult T-cell lymphoma and leukemia (HTLV1+), A nasal form paragraph extraversio NK/T cell lymphoma, a ***** type T cell lymphoma, a ***** type gamma-delta T cell lymphoma, A T cell and spontaneous killer cell nature tumors, such as a hypodermic phlegmon Mr. T cell lymphoma, mycosis fungoides / Sezary syndrome, an anaplasia nature large-sized cell lymphoma (T/null cell, a primary skin undifferentiated type), a peripheral T cell lymphoma that does not belong to a section at others, and a blood vessel immunoblast T cell lymphoma; Hodgkin lymphomas, such as a **** lymphocyte superior Hodgkin lymphoma and a classical Hodgkin's lymphoma (a tuberous sclerosis Hodgkin lymphoma (grades 1 and 2), a lymphocyte rich classical Hodgkin's lymphoma, a mixed cellularity Hodgkin's lymphoma, a lymphocyte depletion Hodgkin lymphoma) (Hodgkin's disease);

Although ** can be mentioned, it is not limited in particular. [0051]

The SHP1 gene-product fixed-quantity process in this invention, Especially if it is the method of doing either [at least / a fixed quantity of] SHP1 protein in a sample sample, or SHP1mRNA, are not limited, but specifically, The method (mRNA assay) of quantifying SHP1mRNA can be conveniently used by detecting the manifestation of mRNA of the method (protein assay) of quantifying SHP1 protein using SHP1 antibody which uses SHP1 protein as an antigen, and SHP1 gene.

[0052]

First, as the more concrete technique of the above-mentioned protein assay, The western blotting method or enzyme-labeled antibody technique (Immunochemistry) using SHP1 antibody (an immunohistochemical method, an immunocytochemistry method, the ELISA (enzyme-linked immunosorbent assay) method) can be mentioned.

[0053]

SHP1 antibody used with the above-mentioned protein assay, It is not what will be limited especially if it is an antibody which recognizes at least a part of structures of SHP1 protein of having the structure shown in <u>drawing 22</u>, <u>drawing 23</u>, and the array number 4, as an antigenic determinant, and can detect SHP1 protein certainly immunologically, It may be a polyclonal antibody and may be a monoclonal antibody. [0054]

The SHP1 above-mentioned antibody may be conventionally manufactured by a publicly known method, and SHP1 commercial antibody may be used for it. As a manufacturing method of

SHP1 antibody, if it is a monoclonal antibody, the technique produced by the hybridoma with which it makes it come to unite the mouse spleen lymphocytes which carried out immunity in SHP1 protein, and the marrow cells of a mouse will be mentioned, for example. If the SHP1 above-mentioned antibody is a polyclonal antibody, the technique refined from the immune serum of the rabbit which carried out immunity in SHP1 protein will be mentioned. #SH-PTP1(D-11):sc7289 and #SH-PTP1(C-19):sc287 (product made from Santa Cruz Biotechnology Inc.), as SHP1 commercial antibody, # anti SHPTP (06117) and #anti mouse SHPTP (05281) (Product made from Upstate Biotechnology Inc.) etc. -- it is mentioned.

The enzyme-labeled antibody technique (an immunohistochemical method, an immunocytochemistry method, the ELISA method) using the SHP1 above-mentioned antibody, A conventionally publicly known method (for example, it, and) [Kazuo-Nakane/ "enzyme-labeled-antibody-technique"-Keiichi-Watanabe and /-] Interdisciplinarity plan publication (Showa 61) and Brown R.W. et al: Modern Pathol.199;8 (5) The method currently indicated by articles, such as 515-20 (1995), can be used conveniently, Neither in particular the concrete process, nor reagents, conditions, etc. are limited. [0056]

Similarly the western blotting method using the SHP1 above-mentioned antibody, a conventionally publicly known method (for example, editing besides "experiment operation blotting method" Yoshiyuki Hino.) a soft science company (Showa 62) and Towbin H. et al: Proc.Natl.Acad.Sci.USA76-4350 (1979), etc. -- the method currently indicated by literature being used conveniently and, Neither in particular the concrete process, nor reagents, conditions, etc. are limited.

[0057]

By using the above-mentioned protein assay, SHP1 protein will be quantified using an antigenantibody reaction. Therefore, it becomes possible to detect hematopoietic organ tumor cells on a high unique target by a simple mechanism.

[0058]

Next, as the more concrete technique of the above-mentioned mRNA assay, The method of detecting the manifestation of mRNA of SHP1 gene using the polynucleotide which has an overall length, or its part and homology of a base sequence of the SHP1 gene cDNA shown in the array number 3 (refer to <u>drawing 21</u>) is mentioned, More specifically, they are a northern blotting method, a reverse transcription polymerase chain reaction method (RT-PCR), a real-time reverse transcription polymerase chain reaction method (real time RT-PCR), or RNA in. A situ hybridization can be mentioned.

[0059]

The above-mentioned northern blotting method, RT-PCR, real time RT-PCR, And RNA in A method that any method of a situ hybridization is conventionally publicly known. for example," - Molecular cloning"a laboratory manual, Sambrook J., Russell DW., and Cold Spring Harbor Lab Press. (2001). "Current protocols in molecular biology"edited by Ausubel FM et al. and John Wiley & Sons Inc. (2001) etc. -- the method currently indicated by literature. It can use conveniently and neither in particular the concrete process, nor reagents, conditions, etc. are limited.

[0060]

The above-mentioned northern blotting method and RNA in situ Theoretically in hybridization, the overall length of cDNA of SHP1 gene shown in the array number 3 or its part can be used as

a probe. The oligonucleotide which has theoretically the part and homology of cDNA of SHP1 gene shown in the array number 3 also by RT-PCR or real time RT-PCR can be used as a primer. The primer pairs specifically shown in Example 3 mentioned later or Example 4, for example can be used.

[0061]

So, what is necessary is just to detect the manifestation of mRNA of SHP1 gene in mRNA assay using the polynucleotide which has an overall length, or its part and homology of a base sequence of the SHP1 gene cDNA shown in the array number 3. [0062]

Since SHP1mRNA which is a transcript of SHP1 gene will be quantified by using the above-mentioned mRNA assay, a mechanism simple by using the polynucleotide which has cDNA and homology of SHP1 gene as a probe or a primer -- quickness -- high -- it becomes possible specific and to detect hematopoietic organ tumor cells to high sensitivity.

[0063]

The SHP1 gene-methylation check process in this invention, Especially if it is the method that methylation of the CpG island included in the base sequence of SHP1 gene obtained from a sample sample can be checked, are not limited, but in this embodiment. For example, the method (the expedient top of explanation and restriction enzyme ascertainment are called hereafter) using a methylation sensitivity restriction enzyme including a gene cutting trial stage, a gene amplification trial stage, and the amount check stage of gene amplification can be used conveniently.

[0064]

With the methylation sensitivity restriction enzyme used by this embodiment. When cytosine is included in the base sequence which serves as a recognition object in double stranded DNA and cytosine in this base sequence is methylated, it will not be limited especially if it is a restriction enzyme which cannot cut the double stranded DNA of this base sequence. [0065]

Specifically as the above-mentioned methylation sensitivity restriction enzyme, HpaII, EagI, or NaeI can be mentioned, for example. Especially, HpaII can be used more preferably. Although it is the endonuclease which HpaII recognizes the base sequence of CCGG and carries out double-stranded-DNA cutting, MspI is known as a restriction enzyme which recognizes the same base sequence and cuts double stranded DNA.

[0066]

As mentioned above, HpaII cannot cut the double stranded DNA of the base sequence of methylated CCGG, but it is not concerned with the existence of methylation, but the base sequence of CCGG is recognized, and MspI can cut double stranded DNA. That is, MspI is a methylation insusceptible restriction enzyme. So, by using HspII and MspI together, it can become possible to use as control for checking cutting of SHP1 gene in a sample sample certainly, and the reliability of the restriction enzyme ascertainment in this embodiment can be further raised so that it may mention later.

[0067]

Thus, in the restriction enzyme ascertainment in this embodiment, it is preferred to use the methylation insusceptible restriction enzyme which recognizes the same base sequence as the methylation sensitivity restriction enzyme to be used as control. Of course, it cannot be overemphasized that the combination of methylation sensitivity and a methylation insusceptible restriction enzyme is not what is limited to above-mentioned HspII-MspI.

[0068]

Next, the check of methylation of SHP1 gene by the SHP1 gene-methylation check process in this embodiment, i.e., restriction enzyme ascertainment, is explained concretely. [0069]

First, it processes with the above-mentioned methylation sensitivity restriction enzyme which recognizes the base sequence which includes for cytosine the genetic material obtained from said sample sample containing a hematopoietic organ cell as a gene cutting trial stage. In this stage, cutting of SHP1 gene contained by processing of a methylation sensitivity restriction enzyme in a genetic material is tried. That is, if the hematopoietic organ cell contained in said sample sample is only a normal cell, SHP1 gene will be cut, but if hematopoietic organ tumor cells are contained, since CG arrangement is methylated, SHP1 gene will not be cut. [0070]

From said sample sample, the method of preparing a genetic material can especially use a publicly known method conventionally, and is not limited. The prepared genetic material should just contain SHP1 gene, and other ingredients may be contained unless restriction enzyme processing, PCR, etc. are checked. So, what is necessary is just a mixture of various DNAs or RNA extracted from the hematopoietic organ cell contained in said sample sample, or other cells. It is not limited in particular for processing by a methylation sensitivity restriction enzyme, and what is necessary is just to set up conditions etc. suitably according to the kind of this methylation sensitivity restriction enzyme, the state of the prepared genetic material, etc. [0071]

Next, PCR is carried out using the primer which amplifies the field which is included in the base sequence of the SHP1 above-mentioned gene as a gene amplification trial stage to the genetic material processed with the above-mentioned methylation sensitivity restriction enzyme, and includes the base sequence by which recognition cutting is carried out in the above-mentioned methylation sensitivity restriction enzyme. In this stage, amplification of only SHP1 gene is tried by carrying out PCR processing of the restriction enzyme treatment object processed with the methylation sensitivity restriction enzyme using the above-mentioned primer. If it is only SHP1 normal gene, since the field inserted into primer pairs is cut, SHP1 gene cannot be amplified, but if SHP1 gene methylated is contained, since the field inserted into the above-mentioned primer pairs is not cut, SHP1 gene will be amplified.

[0072]

What is necessary is just the polynucleotide which amplifies the field which includes the base sequence recognized by the methylation sensitivity restriction enzyme as the above-mentioned primer used in the above-mentioned gene amplification trial stage. So, it is not limited in particular for the design condition of a primer. The primer pairs used by this embodiment fundamentally, Even if there are few fields including the above-mentioned base sequence recognized by the methylation sensitivity restriction enzyme, it is located outside, What is necessary is just a partial base sequence included in the base sequence of SHP1 gene shown in the array number 1 or 2 (refer to <u>drawing 1 - drawing 10 and drawing 11 - drawing 20</u>), or the polynucleotide which has this partial base sequence and complementarity, and it is not limited in particular for that place, size, etc.

[0073]

Next, the quantity of the amplified gene is checked as an amount check stage of gene amplification. In this stage, it is checked whether SHP1 gene has been amplified. If SHP1 gene is amplified, hematopoietic organ tumor cells will be contained in the original sample sample.

[0074]

Although not limited especially as the check method of the existence of SHP1 gene used in the above-mentioned amount check stage of gene amplification, since the technique of checking the amplifying amount of a gene by comparing with a marker using an electrophoresis method is a most common and established technique, it can use preferably. Blotting of the DNA band obtained after electrophoresis may be carried out to a membrane, and it may be detected. 100751

Although not limited especially as the check method of the existence of SHP1 gene used in the above-mentioned amount check stage of gene amplification, Since the technique of checking the amplifying amount of a gene using an electrophoresis method is a most common and established technique after reacting using a methylation positivity and methylation negative control DNA simultaneously with a sample sample, it can use preferably. Blotting of the DNA band obtained after electrophoresis may be carried out to a membrane, and it may be detected. [0076]

SHP1 gene should just be used for the methylation positivity of the SHP1 above-mentioned gene, and methylation negative control DNA, and they are not limited in particular. The DNA solution which has the concentration of the grade which can measure an amplifying amount specifically obtained by processing with a methylation sensitivity restriction enzyme or a methylation insusceptible restriction enzyme can be mentioned.

[0077]

It is desirable, when the same sample sample is processed with a methylation insusceptible restriction enzyme and it is checked in the amount check stage of gene amplification in parallel to processing by a methylation sensitivity restriction enzyme as control in the SHP1 genemethylation check process by restriction enzyme ascertainment. That is, it is dramatically preferred to use the restriction enzyme in which the methylation restriction enzyme [susceptible] which recognizes the same base sequence is known as a methylation sensitivity restriction enzyme in the above-mentioned gene cutting trial stage. This can raise the certainty of methylation of SHP1 gene by restriction enzyme ascertainment. [0078]

The SHP1 satellite LOH check process in this invention, Especially if it is the method that the existence of heterozygosity loss (it abbreviates to Lossof heterozygosity and LOH) of SHP1 gene contained in this sample sample can be checked in the sample sample containing a hematopoietic organ cell, are not limited, but specifically, The Microsatellite marker which puts SHP1 gene, Or about gene polymorphism (polymorphism) like the simple nucleotide polymorphism (single nucleotide polymorphism, SNP) which exists the inside of the above-mentioned SHP gene, and near it. The method of checking LOH in the fragmentation analysis using PCR can be used conveniently.

[0079]

About the Microsatellite marker which exists in the both sides of the SHP1 above-mentioned gene, and the gene polymorphism which exists in SHP1 gene and near it. Although not the thing limited especially but what kind of marker may be used, specifically, D12S336 marker and D12S356 marker can be mentioned, for example. The base sequence of these markers is acquired from the Internet genome database (URL:http://gdbwww.gdb.org./). Among these markers, D12S356 marker exists in the telomere side, and is in the distance of about 4.4 cM(s) from SHP1 gene. On the other hand, D12S356 marker exists in the centromere side, and is in the distance of about 2.4 cM(s) from SHP1 gene.

[0800]

When checking LOH (heterozygosity loss) of SHP1 gene in a sample sample, the sample sample used by a SHP1 satellite LOH check process should just be a sample sample containing a hematopoietic organ cell. What is necessary is just to conduct Microsatellite analysis which detects at least one overall length of each above-mentioned marker, or its part by an PCR reaction, as shown in Example 6 mentioned later although the concrete method in particular of LOH is not limited. In particular the conditions besides an PCR reaction at this time are not limited, either, and as a primer for PCR, For example, what is necessary is for what is necessary just to be a primer which can detect at least some of D12S336 markers or D12S356 markers, and just to set up conditions relevant also about other conditions suitably.

[0081]

The sample sample used by the SHP1 satellite LOH check process in this invention will not be limited especially if it is a sample sample containing a hematopoietic organ cell. As contrast, the sample sample in particular to be used is not limited, either, the sample obtained after complete hematological remission may be used, and other normal tissue cells may be used.

[0082]

Thus, it becomes possible to detect hematopoietic organ tumor cells more certainly by a simple mechanism by checking LOH of SHP1 gene using gene polymorphism, such as a Microsatellite marker and SNP.

[0083]

Although the example which checked LOH of SHP1 gene using a Microsatellite marker and gene polymorphism is given in this embodiment, If this invention is a method which it is not limited to this and LOH of SHP1 gene can check, it cannot be overemphasized that what kind of method may be used.

[0084]

Next, a desirable example of the detecting method concerning this embodiment is explained more concretely.

[0085]

First, at least SHP1 protein contained by the SHP1 gene-product fixed-quantity process in a sample sample using the technique mentioned above or SHP1mRNA either is quantified. When SHP1 protein quantified in this process is decreasing in number more nearly substantially than a standard or SHP1 gene product is hardly revealed, a possibility that hematopoietic organ tumor cells are contained in the sample sample becomes high.

[0086]

Next, methylation of the CpG island included by said restriction enzyme ascertainment by a SHP1 gene-methylation check process in the base sequence of SHP1 gene in the genetic material prepared from the sample sample is checked. In the following explanation, the example which used said HpaII as a methylation sensitivity restriction enzyme is given. Although the base sequence of CCGG is recognized to have mentioned above, HpaII is preferably used, in order that the methylation insusceptible restriction enzyme MspI may recognize the same base sequence.

[0087]

So, in a gene cutting trial stage, the genetic material obtained from the above-mentioned sample sample is processed by HpaII. Simultaneously, it is desirable when the same genetic material is processed by MspI. By this, positive control that a CCGG base sequence is cut can be obtained. [0088]

Next, although it shifts to a gene amplification trial stage, in this step, the primer for PCR is previously set up across the recognition site (CCGG) of HpaII/MspI from the base sequence (refer to the array numbers 1 and 2, <u>drawing 1 - drawing 10</u> and <u>drawing 11 - drawing 20</u>) of SHP1 gene. The primer pairs specifically shown in Example 1 mentioned later or Example 2, for example are used.

[0089]

Using the above primers, PCR is carried out to the genetic material processed by HpaII, it is the amount check stage of gene amplification, and the amplifying amount of an PCR product is checked, for example by electrophoresis. Since HpaII cannot be cut if SHP1 methylated gene is in a genetic material, the PCR product of the target size is detectable by PCR. On the other hand, without SHP1 methylated gene, DNA is cut by HpaII and an PCR product cannot be detected. [0090]

Thus, if the above-mentioned restriction enzyme ascertainment is used, after trying cutting of SHP1 gene contained in the genetic material obtained from the sample sample using the methylation sensitivity restriction enzyme and amplifying using PCR further, the amplifying amount of the PCR product acquired can be checked. So, if even a little SHP1 genes are obtained from a sample sample, it is possible to detect methylation of SHP1 gene. Therefore, even if hematopoietic organ tumor cells exist very much in a sample sample only in the minute amount, it is promptly high detection sensitivity, and it becomes possible to detect hematopoietic organ tumor cells on a high unique target moreover.

[0091]

There is that no other process (process) and other stages (step) may be included in the abovementioned detecting method explained by this embodiment also until it says. For example, in the SHP1 gene-methylation check process, in order to advance a restriction enzyme reaction and an PCR reaction smoothly, the refined stage which refines the genetic material etc. which were obtained may be included.

[0092]

The detection kit for enforcing not only the hematopoietic organ tumor cell detection method mentioned above but this detecting method is contained in this invention. Specifically, the composition containing said SHP1 antibody, said methylation sensitivity restriction enzyme, said each primer, said SHP1 gene positivity, methylation negative control DNA, etc. can be mentioned. It is desirable when it divides into the combination of (1) above-mentioned SHP1 antibody and (2) methylation-sensitivity restriction enzyme, the primer for PCR, and said SHP1 gene positivity and methylation negative control DNA especially, and either [at least] (1) or (2) are contained. Whichever of the turn of a SHP1 gene-product fixed-quantity process and a SHP1 gene-methylation check process may be the point.

[0093]

Other various reagents may be contained in the above-mentioned detection kit if needed. For example, at least one side of reagents for a restriction enzyme reaction, such as reagents for an PCR reaction, such as a nucleotide monomer, polymerase, and a buffer, and a buffer, may be contained.

[0094]

The reagent etc. which are used for every process or stage are explained more concretely. First, in a gene product fixed-quantity process, even if it is any of an enzyme-labeled antibody technique and a western blotting method in the case of protein assay, SHP1 antibody and its detecting reagent are used at least. In the case of mRNA assay, they are a RT-PCR assay and real

time. When using a RT-PCR assay, the primer for SHP1cDNA detection and a Taq DNA polymerase reaction reagent are used at least. [0095]

Next, in the SHP1 gene-methylation check process in this embodiment, in order to check methylation with a methylation sensitivity restriction enzyme, a methylation sensitivity restriction enzyme, methylation insusceptible restriction enzymes, and these reaction reagents are first used at least in a gene cutting trial stage. Next, in a gene amplification trial stage, a primer, a Taq DNA polymerase reaction reagent, and SHP1 gene-methylation positive DNA for system studies are used at least. Next, in the amount check stage of gene amplification, the reaction product which used the SHP1 gene-methylation positivity and methylation negative control DNA can be used at least as control of electrophoresis.

Thus, in the detection kit concerning this invention, in order to enforce the hematopoietic organ tumor cell detection method mentioned above, desirable drugs, a specimen, etc. are contained. Therefore, the hematopoietic organ tumor cell detection method concerning this invention can be enforced easily and simply by using the above-mentioned detection kit, and it becomes possible to use this invention on industrial levels, such as clinical laboratory test industry and pharmaceutical industry.

[0097]

[Embodiment 2]

It will be as follows if other gestalten of the operation in this invention are explained based on drawing 25 thru/or drawing 47. This invention is not limited to this. The explanation of explanation which overlaps with Embodiment 1 for convenience is omitted suitably. [0098]

Although the restriction enzyme ascertainment which uses a methylation sensitivity restriction enzyme for a SHP1 gene-methylation check process was used in said Embodiment 1, This invention is not limited to this and the method (the expedient top of explanation and a DNA modification method are called hereafter) of embellishing DNA using a bisulfite including a gene ornamentation stage and a methylation cytosine content judging stage can be conveniently used for it by this embodiment, for example.

Cytosine will be changed into uracil if DNA is processed by a bisulfite (Bisulfite). As shown in drawing 25, cytosine is specifically sulfonated by the bisulfite (Sulphonation), Furthermore it is deaminated by hydrolysis (Hydrolytic deamination) and is further changed into uracil by the desulfonation (Alkali desulphonation) under alkali existence. This uracil places and changes to thymine after PCR. On the other hand, the methylated cytosine (5'-methylcytosine) is not changed by a bisulfite. So, in this embodiment, using the difference in the base sequence after this bisulfite processing, the existence of methylation of SHP1 gene is detected so that it may mention later.

[0100]

Next, the check of methylation of SHP1 gene by the SHP1 gene-methylation check process in this embodiment, i.e., a DNA modification method, is explained concretely. [0101]

First, the genetic material obtained from said sample sample containing a hematopoietic organ cell as a gene ornamentation stage is processed by a bisulfite. In this stage, since only the cytosine which is not methylated is changed into uracil as mentioned above, if bisulfite

processing of the DNA is carried out, as shown in <u>drawing 26</u>, so, the methylated cytosine (M enclosed with a figure middle circle shows) remains with cytosine, for example, but. The cytosine which is not methylated is changed into uracil (U). [0102]

Especially as a bisulfite used in the above-mentioned gene ornamentation stage, although not limited, sodium bisulfite (it is also called $Na_2S_2O_5$, the sodium metabisulfite, sodium disulfite, or sodium pyrosulfite) can be used conveniently, for example. Urea may be used together with a heavy sulfurous acid compound.

[0103]

Next, the existence of cytosine in the base sequence of SHP1 gene contained in the genetic material processed by the bisulfite as a methylation cytosine content judging stage is judged. That cytosine is contained in SHP1 gene in a bisulfite treatment object, the methylated cytosine will be contained in SHP1 gene before processing. So, if cytosine exists, hematopoietic organ tumor cells will be contained in the original sample sample. [0104]

Although not limited especially as a method of judging the existence of cytosine in the base sequence of SHP1 gene carried out in the above-mentioned methylation cytosine content judging stage, specifically, 1) Which technique can be preferably used at least among the way PCR detects methylation cytosine, the way the determination of the base sequence of a gene detects 2 methylation cytosine, or the method of identifying the base sequence containing 3 methylation cytosine.

[0105]

More specifically, methylation specific PCR (Methylation Specific PCR) can be first mentioned as a way PCR detects 1 methylation cytosine. [0106]

The above-mentioned methylation specific PCR method is specific to methylated DNA, and sets up as a primer a base sequence including CG arrangement. If the methylated cytosine exists, amplification will become possible by PCR, and SHP1 gene so methylated can be detected. [0107]

A publicly known method (for example, the method of Proc. Natl. Acad. Sci. USA 93, 9821-9826 (1996), etc. currently indicated by literature) can be conventionally used for the above-mentioned methylation specific PCR method conveniently, and The concrete process and reagents, Conditions in particular are not limited. In the refining processes of DNA, if the primer which could use the method etc. which used the ethanol precipitation method and the Glassbeads method, and carried out the fluorescence label is used, detection of PCR can be made easy. [0108]

Next, in sequencing of the way, i.e., SHP1 gene, the determination of the base sequence of two genes detects methylation cytosine, a primer is set as the field which does not include CG arrangement, and PCR is carried out. In the PCR product acquired, that (it exists with CG arrangement) by which it is methylated, and the thing (changed into TG arrangement) which is not methylated may be contained. Existence of CG arrangement, i.e., methylation, is considered by carrying out sequencing of this.

[0109]

A method that sequencing of the SHP1 above-mentioned gene is also conventionally publicly known. (For example, the method currently indicated by the literature of Proc. Natl. Acad. Sci. USA 89, 1827-1831 (1992), etc.) can be used conveniently, and neither in particular the concrete

process, nor reagents, conditions, etc. are limited. It is also possible to use the primer which has specific arrangement (CG arrangement is included) for methylated DNA as the above-mentioned primer.

[0110]

Since PCR is used, this method can also detect methylation of SHP1 gene, if even a little SHP1 genes are obtained from a sample sample. Therefore, even if hematopoietic organ tumor cells exist very much in a sample sample only in the minute amount, it becomes possible to detect hematopoietic organ tumor cells on a high unique target by high detection sensitivity. Since concrete arrangement is determined by using sequencing, it also becomes possible to clarify the grade of methylation more.

[0111]

Next, as a method of identifying the base sequence containing 3 cytosine, the Ms-SnuPE method, the bisulfite SSCP method, the methyl light method, a fluorescence dissolution curvilinear analysis method, the COBRA method, etc. can be mentioned.

[0112]

The describing [above] Ms-SnuPE (Methylation-sensitive Single Nucleotide Primer Extension) method is a method of carrying out PCR using a specific primer to methylated DNA. However, since the existence of methylation in the field inserted into the primer is not known, the polynucleotide which adjoins CG arrangement to detect is created and it is made to anneal with an PCR product. If ³²P-dCTP is incorporated when DNA is compounded under existence of radioisotope, the cytosine methylated since it is CG arrangement there will exist. Since it is TG arrangement there, it means that methylation was not carried out on the other hand if ³²P-dTTP was incorporated when DNA is compounded.

[0113]

A publicly known method (for example, the method of Nucleic Acids Research25, 2529-2531 (1997), etc. currently indicated by literature) can be conventionally used for the describing [above] Ms-SnuPE method conveniently, Neither in particular the concrete process, nor reagents, conditions, etc. are limited.

[0114]

The describing [above] bisulfite SSCP (Bisulfite-SSCP) method does not understand the existence of methylation in the field inserted into the primer, either, although it is the method of carrying out PCR using a specific primer to methylated DNA. Then, electrophoresis of the PCR product is carried out after denaturalizing to single-strand DNA using the SSCP (Single Strand Conformational Polymorphism) method, and the grade of methylation of SHP1 gene is judged from the difference in the mobility of single-strand DNA.

[0115]

The describing [above] bisulfite SSCP method can also use conveniently conventionally a publicly known method (for example, the method of Electrophoresis 21, 904-908 (2000), etc. currently indicated by literature), and neither in particular the concrete process, nor reagents, conditions, etc. are limited.

[0116]

To others, the methyl light (Methyl-light) method, the fluorescence dissolution curvilinear analyzing (FluorescenceMelting Curve Analysis) method, etc. are mentioned. No these methods also understand the existence of methylation in the field inserted into the primer, although it is the method of carrying out PCR using a specific primer to methylated DNA. then -- the field which wants to investigate the inside -- methylation -- the quantity of methylation in the above-

mentioned PCR product is judged by creating specific polynucleotide and examining how much [the PCR product which this methylation specific polynucleotide made the single strand, and] an annealing (2 chain polymerization) reaction is carried out. [0117]

The methyl light describing [above] method specifically, for example Nucleic Acids Research 28 (8), E32 (2000), etc. -- the method currently indicated by literature -- the above-mentioned fluorescence dissolution curvilinear analysis -- concrete -- Clinical Chemistry47 and 1183-1189 (2001), etc. -- the method currently indicated by literature can be used conveniently. [0118]

Since PCR is used, the all directions method mentioned above can detect methylation of SHP1 gene, if even a little SHP1 genes are obtained from a sample sample. Therefore, even if hematopoietic organ tumor cells exist very much in a sample sample only in the minute amount, it becomes possible to detect hematopoietic organ tumor cells on a high unique target by high detection sensitivity.

[0119]

The describing [above] COBRA method () [Combined Bisulfite RestrictionAnalysis and] Or if CGCG arrangement has received [being called Bisulfite PCR followed byrestriction analysis etc. and] methylation, for example, after heavy sulfite treating remains with CGCG arrangement, but. It will be changed into TGTG arrangement if not methylated. Then, by using the restriction enzyme etc. which cut only the above-mentioned CGCG arrangement, the band pattern on electrophoretic gel can be analyzed, and the existence of methylation of SHP1 gene can be judged and quantified.

[0120]

The describing [above] COBRA method can also use conveniently conventionally a publicly known method (for example, the method of Nucleic Acids Research 25-2532-2534 (1997) etc. currently indicated by literature), Neither in particular the concrete process, nor reagents, conditions, etc. are limited. Of course, the advantage by PCR mentioned above is not only acquired, but [since PCR is used also by this method,] since restriction enzyme processing and electrophoresis are used, if even the analysis of a band pattern is clarified, there is an advantage that methylation of SHP1 gene can be checked easily.

Thus, by the DNA modification method in this embodiment, although PCR is used in the methylation cytosine content judging stage, the designing method of a primer used by this PCR is explained below.

[0122]

As mentioned above, if bisulfite processing of the DNA is carried out, cytosine will be changed into uracil, but the methylated cytosine is saved, without being changed. Here, the cytosine which may receive methylation by intracellular is cytosine (C) of CG arrangement (5'-CG-3') located in a line with CG from the 5' arrangement side. Therefore, all cytosine other than the above-mentioned CG arrangement will be changed into thymine (T) by bisulfite processing. Then, all the CG arrangement changes the base sequence of SHP1 gene as what received methylation, and sets up a primer. Uracil in DNA will be recognized as thymine and will be replaced by thymine by PCR.

[0123]

First, the conditions about the DNA strand which plans a primer are set up. In the base sequence of SHP1 gene, either a sense strand or an antisense strand assumes the arrangement from which

all cytosine in other base sequences was changed into thymine noting that only the abovementioned CG arrangement receives methylation. [0124]

. The base sequence shown in <u>drawing 27 - drawing 36</u>, and the array number 5 specifically corresponds to the sense strand of the genomic DNA (wild type) of SHP1 gene shown in <u>drawing 1 - drawing 10</u>, and the array number 1. It is a base sequence (it is hereafter considered as sense strand conversion arrangement on [of explanation] expedient) after bisulfite processing, . Correspond for the base sequence shown in <u>drawing 37 - drawing 46</u>, and the array number 6 to consider it as the antisense strand of the genomic DNA (wild type) of SHP1 gene shown in <u>drawing 11 - drawing 20</u>, and the array number 2. It is a base sequence (it is hereafter considered as antisense strand conversion arrangement on [of explanation] expedient) after bisulfite processing. These sense strand conversion arrangement and antisense strand conversion arrangement become less complementary by bisulfite processing.

The base sequence of <u>drawing 27</u> - <u>drawing 36</u>, the array number 5 and <u>drawing 37</u> - <u>drawing 46</u>, and the array number 6, When it is assumed that CG arrangement is methylated 100%, it is a base sequence as what received bisulfite processing, and since it is not thought that 100% of methylation arises in a cell actually, it illustrates as a base sequence as a possibility that it can detect in this invention.

[0126]

And a forward primer (FW primer) and a reverse primer (RV primer) are created to the (I) above-mentioned sense strand conversion arrangement, or FW primer and RV primer are created to the (II) above-mentioned antisense strand conversion arrangement. In this case, primer arrangement differs also at the same place, respectively.

[0127]

Next, the conditions about the field which plans a primer are set up. (i) In order to amplify what created the primer to the base sequence including CG arrangement, or was (ii)-methylated in order to amplify only methylated DNA by PCR directly, and the thing which is not carried out by PCR fair, create a primer to the arrangement which does not include CG field. When it is (ii), the method of sequencing or others is enforced later and methylation is judged. [0128]

Therefore, there are four kinds of designing methods which multiplied condition (i) and condition (ii) about condition (I) about the above-mentioned DNA strand and condition (II), and a field in the design of the primer used by a DNA modification method.

[0129]

Here, in (i), if the place of the primer has received methylation with sufficient convenience, it will be detected, but when only the adjacent area instead of a place have received methylation, it becomes undetectable although methylation exists. Then, methylation of SHP1 gene is certainly detectable by authorizing the existence of the methylation in the field which was not concerned with the existence of methylation but was surrounded by each primer after amplification by PCR like (ii), i.e., CG arrangement. Therefore, the information of not only the place of the primer for detection but a gene sequence itself becomes important for the judgment of methylation of SHP1 gene in this embodiment.

[0130]

If CG arrangement is not methylated, will be changed into TG arrangement by bisulfite processing, but. The primer (Unmethylated primer) created to a base sequence including this TG

arrangement can be used as control proving existence of DNA which has not received methylation. When bisulfite processing is insufficient, SHP1 gene of the wild type from which cytosine is not changed into uracil will mix. Then, the primer (Wild type primer) which has a wild type base sequence can be used as control of whether bisulfite processing was made thoroughly enough.

[0131]

In the methylation cytosine content judging stage mentioned above. The method same to the check of the gene amplified by PCR as the amount check stage of gene amplification in said Embodiment 1, For example, by comparing with a marker using an electrophoresis method, the amplifying amount of a gene is checked or the technique of having ****ed enough blotting of the DNA band further obtained after electrophoresis to the membrane, and carrying out it is mentioned. Of course, it is not limited to these techniques, and a publicly known technique can be conventionally used conveniently also about the above-mentioned electrophoresis method or the method of blotting, and it is not limited in particular.

[0132]

As long as it puts in another way, the amount check stage of gene amplification as well as the case where it is based on the restriction enzyme ascertainment in said Embodiment 1 may be included also by the SHP1 gene-methylation check process by the DNA modification method in this embodiment.

[0133]

Next, a desirable example of the detecting method concerning this embodiment is explained more concretely.

[0134]

First, either [at least] SHP1 protein contained by the SHP1 gene-product fixed-quantity process in a sample using the technique mentioned above or SHP1mRNA is quantified. When SHP1 protein quantified in this process is decreasing in number more nearly substantially than a standard or SHP1 gene product is hardly revealed, a possibility that hematopoietic organ tumor cells are contained in the sample sample becomes high.

[0135]

Next, methylation of the CpG island included by said DNA modification method by a SHP1 gene-methylation check process in the base sequence of SHP1 gene in the genetic material prepared from the sample is checked. specifically, the genetic material obtained from the above-mentioned sample sample is come out of and processed in a gene ornamentation stage using sodium bisulfite.

[0136]

Next, although it shifts to a gene amplification trial stage, in this step, the primer for PCR is set up based on the designing method of the primer mentioned above. [0137]

By methylation specific PCR, as shown in <u>drawing 47</u> (a), specifically, suppose that CG arrangement of wild type DNA has methylation 100% supposing wild type DNA (for figure Nakagami, the bottom is an antisense strand at a sense strand) of 23 base pairs. When bisulfite processing is carried out, it becomes in this case, less complementary in a sense strand and an antisense strand, as shown in <u>drawing 47</u> (b). Then, as shown in <u>drawing 47</u> (c) or (d), FW primer and RV primer are created to a sense strand or an antisense strand.

[0138]

In above-mentioned methylation specific PCR, the primer pairs specifically shown in Example 4

mentioned later or Example 5, for example are used as a primer for PCR. Using the above primers, methylation specific PCR is carried out to the genetic material processed with sodium bisulfite, for example, the amplifying amount of an PCR product is checked by electrophoresis. [0139]

Thus, if the describing [above] DNA modification method is used and the genetic material obtained from the sample using the bisulfite will be processed, cytosine in a base sequence will be changed into uracil, but the methylated cytosine is not changed. Therefore, methylation of SHP1 gene is detectable only by judging whether cytosine is contained in the base sequence of SHP1 gene after a gene ornamentation stage. Therefore, it becomes possible quick by a simple mechanism and to detect hematopoietic organ tumor cells on a high unique target.

[0140]

Next, either [at least] SHP1 protein contained by the SHP1 gene-product fixed-quantity process in a sample sample using the technique mentioned above or SHP1mRNA is quantified. When SHP1 gene products quantified in this process are decreasing in number more nearly substantially than a standard or are hardly revealed, a possibility that hematopoietic organ tumor cells are contained in the sample sample becomes high.

[0141]

Like the detecting method of said Embodiment 1, there is that no other process (process) and other stages (step) may be included in the above-mentioned detecting method explained by this embodiment also until it says.

[0142]

The detection kit for enforcing not only the hematopoietic organ tumor cell detection method mentioned above but this detecting method is contained in this invention. The composition which specifically contains the bisulfite refined to the gene processing level, said primer, and said SHP1 antibody can be mentioned. In the detection kit concerning this invention, the above-mentioned bisulfite, a primer, and SHP1 antibody That is, (1) above-mentioned SHP1 antibody and (2) bisulfites, The primer for a judgment of the existence of cytosine in the base sequence of SHP1 gene contained in the genetic material processed by this bisulfite, And when it divides into the primer for PCR which detects the overall length of a base sequence, or its part of SHP1 gene cDNA shown in the (3) array number 3, it is preferred that any at least one of (1), (2), and (3) is included.

[0143]

The probe for Northern blotting which has an overall length, or its part and homology of a base sequence of the SHP1 gene cDNA shown in the array number 3 in the above-mentioned detection kit, Or the marker for electrophoresis using the methylation positivity of a restriction enzyme and SHP1 gene and methylation negative control DNA which recognize the base sequence containing cytosine may be included, At least one side of reagents for a restriction enzyme reaction, such as reagents for an PCR reaction, such as a nucleotide monomer, polymerase, and a buffer, and a buffer, may be contained. [0144]

The reagent etc. which are used for every process or stage are explained more concretely. First, in a gene product fixed-quantity process, since it is the same as that of what was mentioned as the example by said Embodiment 1, the explanation is omitted.

[0145]

Next, in the SHP1 gene-methylation check process in this embodiment, in order to check

methylation by bisulfite processing, reagents, such as various bisulfites, are first used at least in a gene ornamentation stage. Next, in a methylation cytosine content judging stage, when using how PCR detects methylation cytosine, methylation arrangement specific primers and a TaqDNA polymerase reaction reagent are used at least. moreover -- the way the determination of the base sequence of a gene detects methylation cytosine, or the method of recognizing the base sequence containing cytosine -- each -- publicly known reagents are used according to a concrete method.

[0146]

Thus, in order to enforce the hematopoietic organ tumor cell detection method mentioned above like [the detection kit concerning this embodiment] the detection kit of said Embodiment 1, desirable drugs, a specimen, etc. are contained. Therefore, the hematopoietic organ tumor cell detection method concerning this invention can be enforced easily and simply by using the above-mentioned detection kit, and it becomes possible to use this invention on industrial levels, such as clinical laboratory test industry and pharmaceutical industry.

[0147]

This invention is not what is limited to each embodiment mentioned above, It cannot be overemphasized that it is contained in the technical scope of this invention also about the embodiment obtained by embodiment which various change is possible and is different in the range shown in the claim combining suitably the technical means indicated, respectively. [0148]

EXAMPLE

[Example]

Hereafter, the concrete example of this invention is described based on <u>drawing 48</u> thru/or <u>drawing 52</u>. This invention is not limited to this.

[0149]

[Example 1]

In accordance with Towbin H. et al:Proc.Natl.Acad.Sci.USA76-4350 and the method currently indicated by (1979), Western blotting was carried out using the sample sample containing a spontaneous killer cell lymphoma. #SH-PTP1(D-11):sc7289 (product made from Santa Cruz Biotechnology Inc.) was used as SHP1 antibody (a SHP1 gene-product fixed-quantity process and protein assay).

[0150]

Then, it shifted to the SHP1 gene-methylation check process. First, the genetic material prepared from the above-mentioned sample sample was processed in 37 ** 4 hours, using HpaII as a methylation sensitivity restriction enzyme (gene cutting trial stage).

Next, the genetic material processed by HpaII was amplified by PCR (gene amplification trial stage). The Pullar **-*** used at this time was taken as the combination of primer REP-S1 of 19 bases shown in the array number 7 and <u>drawing 48 (a)</u>, and primer REP-AS1 of 20 bases shown in the array number 8 and <u>drawing 48 (b)</u>. When these primer pairs are used, as shown in the array number 9 and <u>drawing 48 (c)</u>, the base sequence of 126 bases from 7441 bases in the arrangement (refer to the array number 1 and <u>drawing 1 - drawing 10</u>) of the sense strand of SHP1 gene to 7566 bases is detected.

[0152]

"# (number)" in the parenthesis in <u>drawing 48</u> (c) shows the position of the base in the sense strand of the SHP1 above-mentioned gene, and the underline part shows the correspondence position of primer REP-S1 and REP-AS1, and the position of the recognition cleavage site of HpaII. Primer REP-AS1 is designed to the arrangement of the antisense strand in the field of the underline part of above-mentioned REP-AS1.

[0153]

Then, after carrying out electrophoresis by agarose gel, blotting of the obtained DNA band was carried out to the nylon membrane, and amplification of SHP1 gene was checked (the amount check process of gene amplification).

[0154]

Next, Towbin H. et al: Western blotting was carried out in accordance with Proc.Natl.Acad.Sci.USA76-4350 and the method currently indicated by (1979). #SH-PTP1(D-11):sc7289 (product made from Santa Cruz Biotechnology Inc.) was used as SHP1 antibody (a SHP1 gene-product fixed-quantity process and protein assay).

[0155]

The hematopoietic organ tumor cells in a sample sample were detected from the result of the above-mentioned SHP1 gene-methylation check process and a SHP1 gene-product fixed-quantity process.

[0156]

[Example 2]

Primer REP-S2 of 21 bases shown in the array number 10 and <u>drawing 49</u> (a) as primer pairs in a gene amplification trial stage, The existence of the hematopoietic organ tumor cells in a sample sample was detected like said Example 1 except having used the combination of primer REP-AS2 of 21 bases shown in the array number 11 and <u>drawing 49</u> (b).

[0157]

When the above-mentioned primer pairs are used, as shown in the array number 12 and <u>drawing 49 (c)</u>, the base sequence of 227 bases from 6858 bases in the arrangement (refer to the array number 1 and <u>drawing 1 - drawing 10</u>) of the sense strand of SHP1 gene to 7084 bases can be detected.

[0158]

"# (number)" in the parenthesis in <u>drawing 49</u> (c) shows the position of the base in the sense strand of the SHP1 above-mentioned gene, and the underline part shows the correspondence position of primer REP-S2 and REP-AS2, and the position of the recognition cleavage site of HpaII. Primer REP-AS2 is designed to the arrangement of the antisense strand in the field of the underline part of above-mentioned REP-AS2.

[0159]

[Example 3]

The existence of the hematopoietic organ tumor cells in a sample sample was examined like said Example 1 except having carried out the SHP1 gene-product fixed-quantity process using the mRNA assay by RT-PCR.

[0160]

That is, after preparing RNA intracellular [all the] from said sample sample, reverse transcription was carried out with reverse transcriptase. Then, it amplified by PCR using SHP1 specific primer pairs. As the above-mentioned SHP1 specific primer pairs, the combination of primer SHP-PF1 of 23 bases shown in the array number 13 and drawing 50 (a) and primer SHP-

PR1 of 25 bases shown in the array number 14 and <u>drawing 50</u> (b) was used. [0161]

[Example 4]

The existence of the hematopoietic organ tumor cells in a sample sample was examined like said Example 3 (namely, said Example 1) except having carried out the SHP1 gene-product fixed-quantity process using the mRNA assay by real time RT-PCR. As the above-mentioned SHP1 specific primer pairs, primer SHP-LR1 of 20 bases shown in primer SHP-LF1, and the array number 16 and drawing 51 (b) of 20 bases shown in the array number 15 and drawing 51 (a) was used.

[0162]

[Example 5]

Proc. Natl. Acad. Sci. USA 93 and 9821-9826 (1996), Except having carried out the SHP1 genemethylation check process using methylation specific PCR in accordance with the method currently indicated, The existence of the hematopoietic organ tumor cells in a sample sample was examined like said Example 1. Sodium bisulfite was used as a bisulfite.

[0163] As primer pairs in above-mentioned methylation specific PCR, the combination of primer MF2 of 24 bases shown in the array number 17 and <u>drawing 52</u> (a) and primer MR2 of 21 bases shown in the array number 18 and <u>drawing 52</u> (b) can be used. When these primer pairs are used,

as shown in the array number 19 and <u>drawing 52</u> (c), the base sequence of 159 bases from 7037 bases in the arrangement (refer to the array number 1 and <u>drawing 1 - drawing 10</u>) of the sense

strand of SHP1 gene to 7195 bases can be detected. [0164]

"# (number)" in the parenthesis in <u>drawing 52</u> (c) shows the position of the base in the sense strand of the SHP1 above-mentioned gene, and the underline part shows the correspondence position of the primers MF2 and MR2. However, since each above-mentioned primer is designed detect only DNA methylated, the base sequence differs from the base sequence of the above-mentioned underline part for a while. Primer MR2 is designed to the arrangement of the antisense strand in the field of the underline part of above-mentioned MR2. [0165]

[Example 6]

As a sample sample, the bone marrow (BM) sample for diagnosis and the ALL (acute lymphoblastic leukemia) patient's peripheral blood (PB) sample were used. BM sample obtained from the ALL patient contained the blast cell by at least 70% of ratio. The control sample to these sample sample was obtained after the complete hematological remission attained by the chemotherapy.

[0166]

Microsatellite analysis was conducted using the above-mentioned sample sample. the PCR reaction at this time -- the primer by the side of 5' -- 5' -- a label being carried out by - iodoacatamidefluorescein and the system of reaction, It was considered as each dNTP of each primer of 10pmol, the genomic DNA of 40ng, a 1xPCR buffer, and 200microM, and the system of 20microl containing Taq DNApolymerase of 0.5unit. The acquired PCR product is covered over ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA), It analyzed by Genescan Analysis software ver 3.7 (Applied Biosystems).

As a result, as shown in drawing 53 (a) - (b), it turned out that the existence of LOH of SHP1

gene can be checked with D12S336 marker and D12S356 marker. In the result of this example, LOH was observed in 15 examples (79%) with D12S356 marker by the side of a telomere among these markers among 19 cases from which the significant result was obtained. In D12S336 marker by the side of centromere, LOH was observed in six examples (38%) among 16 cases. [0168]

Any result of the example of the above was fully able to detect hematopoietic organ tumor cells from the sample sample. So, even if this invention did not use two or more modalities together, it turned out that hematopoietic organ tumor cells are easily and promptly detectable. [0169]

[Effect of the Invention]

As mentioned above, the hematopoietic organ tumor cell detection method concerning this invention, The SHP1 gene-product fixed-quantity process of quantifying either [at least] SHP1 specific protein or its mRNA into the hematopoietic organ cell contained in the sample sample containing a hematopoietic organ cell, The SHP1 gene-methylation check process which checks the methylation of the CpG island included in the base sequence of SHP1 gene which encodes the SHP1 above-mentioned protein acquired from the above-mentioned sample sample, It is a method including the SHP1 gene LOH check process which checks the existence of heterozygosity loss (LOH) of SHP1 gene contained in the above-mentioned sample sample. [0170]

As a desirable example of the hematopoietic organ tumor cell detection kit concerning this invention, (1) The methylation sensitivity restriction enzyme which recognizes the base sequence which contains in a hematopoietic organ cell SHP1 antibody which uses protein tyrosine phosphatase SHP1 specific protein as an antigen, and (2) cytosine, The primer for PCR which amplifies the field which is included in the base sequence of SHP1 gene and includes the base sequence recognized by the above-mentioned methylation sensitivity restriction enzyme, In the composition which contains at least one side the methylation positivity of the SHP1 abovementioned gene, and among methylation negative control DNAs. Or SHP1 antibody which uses protein tyrosine phosphatase SHP1 protein specific into (1) hematopoietic organ cell as an antigen and the bisulfite refined to (2) gene processing level, The primer for a judgment of the existence of cytosine in the base sequence of SHP1 gene contained in the genetic material processed by this bisulfite, And it is used in order to detect hematopoietic organ tumor cells from the sample sample containing the composition containing any at least one of the primers for PCR which detect the overall length of a base sequence, or its part of SHP1 gene cDNA shown in the (3) array number 3, or a hematopoietic organ cell, The composition containing the primer for PCR which detects at least one overall length of two Microsatellite markers which put protein tyrosine phosphatase SHP1 specific gene between a hematopoietic organ cell, or its part can be mentioned.

[0171]

According to the method or composition of this invention, SHP1 gene expression can be judged using four-fold marker by the maximum called loss of ornamentation and mRNA of gene DNA, protein, and allele. namely, one hematopoietic organ tumor cell called a SHP1 gene-expression fall -- since a specific phenomenon can be checked in four steps, hematopoietic organ tumor cells are detectable by very high singularity. Therefore, hematopoietic organ tumor cells are easily and promptly detectable from a small amount of sample samples containing a hematopoietic organ cell by using this invention.

[0172]

So, if the high sensitivity detection system of the malignant lymphoma and leukemia in this invention is used, The monitoring after the early detection of the hematopoietic organ tumor by a general mass screening, diagnosis, and a therapy and the early detection of a recurrence become possible, and it also becomes possible to use this invention for prediction etc. of the crisis risk in blood relationship persons, such as a family who showed the symptoms of these diseases. As a result, the effect of becoming possible to use this invention on industrial levels, such as clinical laboratory test industry and pharmaceutical industry, is done so.

[0173]

(110) Japan Science and Technology Corporation

<120> Hematopoietic malignant cell-d
lignant cell-detection kit

<130> Y2002-P331

 $\langle 170 \rangle$ Patent In Ver. 2.1

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(210) 8

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 $\langle 211 \rangle 126$

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<211>20

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<213> Artificial Sequence

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⟨211⟩ 24

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<211>21

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<213> Artificial Sequence

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<223> Description of Artificial Sequ Synthesized Primer Sequence

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<210> 19

<211> 159

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<213> Homo sapiens

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is a base sequence figure showing the base sequence of the sense strand of the SHP1 gene genomic DNA used with the hematopoietic organ tumor cell detection method concerning this invention.

[Drawing 2]It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in drawing 1.

[Drawing 3]It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in drawing 1 and drawing 2.

[Drawing 4] It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in <u>drawing 1 - drawing 3</u>.

[Drawing 5] It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in drawing 1 - drawing 4.

[Drawing 6] It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in drawing 1 - drawing 5.

[<u>Drawing 7</u>]It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in <u>drawing 1</u> - <u>drawing 6</u>.

[Drawing 8]It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in <u>drawing 1</u> - <u>drawing 7</u>.

[Drawing 9]It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in <u>drawing 1 - drawing 8</u>.

[Drawing 10] It is an arrangement figure showing a continuation of the base sequence of the sense strand in the genomic DNA of SHP1 gene shown in drawing 1 - drawing 9.

<u>[Drawing 11]</u>It is a base sequence figure showing the base sequence of the antisense strand in the genomic DNA of SHP1 gene used with the hematopoietic organ tumor cell detection method concerning this invention.

[Drawing 12] It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u>.

[Drawing 13] It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u> and <u>drawing 12</u>.

[Drawing 14] It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in drawing 11 - drawing 13.

[Drawing 15] It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in drawing 11 - drawing 14.

[Drawing 16] It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u> - <u>drawing 15</u>.

[Drawing 17] It is an arrangement figure showing a continuation of the base sequence of the

antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u> - <u>drawing 16</u>.

[<u>Drawing 18</u>]It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u> - <u>drawing 17</u>.

[<u>Drawing 19</u>]It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u> - <u>drawing 18</u>.

[<u>Drawing 20</u>]It is an arrangement figure showing a continuation of the base sequence of the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 11</u> - <u>drawing 19</u>.

[<u>Drawing 21</u>]It is a base sequence figure showing the base sequence of cDNA of SHP1 gene used with the hematopoietic organ tumor cell detection method concerning this invention.

[<u>Drawing 22</u>]It is a mimetic diagram showing the outline structure of SHP1 protein used with the hematopoietic organ tumor cell detection method concerning this invention.

[Drawing 23] It is an amino acid sequence figure showing the amino acid sequence of SHP1 protein shown in drawing 22.

[Drawing 24] In the genomic DNA (sense strand) of SHP1 gene shown in <u>drawing 1</u>, it is a base sequence figure showing the part of CG arrangement methylated on a CpG island.

<u>[Drawing 25]</u>It is a chemical reaction explanatory view showing the process in which cytosine is changed into uracil in the heavy sulfite treating used with the hematopoietic organ tumor cell detection method concerning this invention.

<u>[Drawing 26]</u>It is a mimetic diagram showing the state where cytosine is changed into uracil and the methylated cytosine is not changed by the heavy sulfite treating used with the hematopoietic organ tumor cell detection method concerning this invention.

<u>[Drawing 27]</u>It is a base sequence figure showing the base sequence after carrying out bisulfite processing to the sense strand of the SHP1 gene genomic DNA used with the hematopoietic organ tumor cell detection method concerning this invention.

[Drawing 28] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27.

<u>[Drawing 29]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in <u>drawing 27</u> and 28.

[Drawing 30] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27 - drawing 29.

<u>[Drawing 31]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27 - drawing 30.

[Drawing 32]It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27 - drawing 31.

[Drawing 33] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27 - drawing 32.

[Drawing 34] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27 - drawing 33.

[Drawing 35] It is an arrangement figure showing a continuation of the base sequence after

carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in drawing 27 - drawing 34.

<u>[Drawing 36]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the sense strand in the genomic DNA of SHP1 gene shown in <u>drawing 27</u> - <u>drawing 35</u>.

<u>[Drawing 37]</u>It is a base sequence figure showing the base sequence after carrying out bisulfite processing to the antisense strand of the SHP1 gene genomic DNA used with the hematopoietic organ tumor cell detection method concerning this invention.

<u>[Drawing 38]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in drawing 37.

<u>[Drawing 39]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 37</u> and <u>drawing 38</u>.

[Drawing 40] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in drawing 37 - drawing 39.

<u>[Drawing 41]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 37</u> - <u>drawing 40</u>.

[<u>Drawing 42</u>]It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 37</u> - <u>drawing 41</u>.

<u>[Drawing 43]</u>It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in <u>drawing 37</u> - <u>drawing 42</u>.

[Drawing 44] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in drawing 37 - drawing 43.

[Drawing 45]It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in drawing 37 - drawing 44.

[Drawing 46] It is an arrangement figure showing a continuation of the base sequence after carrying out bisulfite processing to the antisense strand in the genomic DNA of SHP1 gene shown in drawing 37 - drawing 45.

[Drawing 47](a) - (d) is a mimetic diagram showing the step of methylation specific PCR used by this invention, respectively.

<u>[Drawing 48]</u>(a) It is a base sequence figure showing the primer for PCR used in Example 1 whose - (b) is an example of operation of this invention, and (c) is a base sequence figure showing the base sequence of SHP1 gene (genomic DNA and sense strand) which the primer for PCR used by (a) - (b) recognizes.

[Drawing 49](a) It is a base sequence figure showing the primer for PCR used in Example 2 whose - (b) is an example of operation of this invention, and (c) is a base sequence figure showing the base sequence of SHP1 gene (genomic DNA and sense strand) which the primer for PCR used by (a) - (b) recognizes.

[Drawing 50](a) It is a base sequence figure showing the primer for RT-PCR used in Example 3

whose - (b) is an example of operation of this invention.

[Drawing 51](a) It is a base sequence figure showing the primer for real time RT-PCR used in Example 4 whose - (b) is an example of operation of this invention.

[Drawing 52](a) Are the primer for methylation specific PCR used in Example 5 whose - (b) is an example of operation of this invention a shown base sequence figure, and (c), (a) It is a base sequence figure showing the base sequence of SHP1 gene (genomic DNA and sense strand) which the primer for methylation specific PCR used by - (b) recognizes.

<u>[Drawing 53]</u>(a) is a figure showing fluorescence in situ hybridization (FISH), and (b) is a figure showing one typical data of the analysis result of heterozygosity loss of SHP1 gene in an ALL patient.

DRAWINGS

[Drawing 1]

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                                                                       1080
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[Drawing 2]

ctategagag gotaaggtga gaggottgot tgagootggg aggtcaaggo tgcagtoago gatgattene conctenent ocasportes openesate apportisto tons 2100 assassassa gasastgaso cagottosta tgotagosag tgaotgggtg tgcaggtgao 2160 attactagot ggagggatca gggaggoott occgaggagg tgacatttga gotgagacco 2220 2280 ggatgaggag gaagaggage tegecatgtg acgtagtgat caagagteaa geatetetegg geagaggaga testeageac asagocetas teteggasea sacasasasa suacastate 2340 2400 cocgiged aggacoutes tesseogram commerces aggagettes accentities agotaggatg itgasagtga assoctgeog agetgaggtg gogcacgtot etgatcocag 2460 2520 cactiteera recognagee remarattec ttemectom cantitamas compocters cascatagag agacoccate totattassa assestactg ggtatgatgg coccagostg 2580 tggtagtoot ageagtitgg gaggotgagg tgggaggate acttgageoc aagagtteaa 2640 gaccaccotg ggcascateg ggagagacot catototact acquotacga stestattac 2700 tectastasa tagotggata tagtggoetg cacctgtggt ctcagttact tggesagotg 2760 aggosggagg atoacctgag ocaaggaggt ogsogotgce gtgagttgga ttgtgacact 2820 goacticago ctgggtgata asgcaagatt ctgtgtcasa sassassasa assagagagg PROTERRED SEVERITORS PERSONALS BARRESTESS PROFESSION ASSESSMENT 2940 OFRICARIAS GENERALS CONFIDENCE OF SERVICES SERVICES SERVICES OF SE 3000 augigacaco cagiogasag aagasaggaa agasaaagaa asagigacaa coggiogasa 3060 gaasaaagaa saagtgacaa coggetggge atggtggete sageetgtaa teecagcaet tregango carecagete estcacrage tongraptic assaccaged trecessest 3180 ggtgasacco tgtotcaact assgatacas aasaasaatt aggotggcac agtggtgego 3240 accigigagi occagciaci agggaggotg aggcaggaga stigotigaa occaggaggo 3300 egsegttena gtgagnogag attgogtnac tgoactocag optgagtgos gogggagagas 3360 otcontotos asessesses assasgeses gamesagtes cascotgott sosgestact 3420 ggogagtitg teggteggte gotocotago cotgetgatt ettgettete acastoatgt 3480 otgoccotgo cocagtgeac atottgtoac tgtoggecco accgatgegg ttoctactga gtottotget coctsetocc gtotstggto attittoctge cangiagott ggeoggeot 3600 cocctggtgc agatttosto ottggtttet cagootggoc ttggatgace otstacages 3660 gggterocca ctotoagame mactitgete cagocacatg getigeteme ggeoaggene 3720 igeocetata sectotatae ataccecte titaccetae cocetatae ciciasana 3780 geacttette eteoacette eateatggge tgtggeagtg coesteeest etgecoogs 3840 cgctgtctgc tgcagtatgg ttgttggggg samgggcsoc mggctooggo gtotgaomge 3900 ogigitate ocception actorises tigigacett gggeratte timecatote 3960

[Drawing 3]

tgagtottag tittetgitte tassatiggg tgaetsacae etactsagta gggitggeet 4020 gaggattaat agtataatgt assagctggc agcactgasa coctgooact taccagcttt 4080 tomostomyt ettigggmen istigitmeg etcettigie eggegggget ietgaggete 4140 agagoagtic cagaactite tacagattat titigoctigi tigogotico agactgocta 4200 4260 tettettete teaccettes tettestete tataetttit asttittiti titttaagae graptiticae tetriticee aggetgrant gegetgreat gateteret cactgeasoe 4320 tocacotcot gagaagetgg gattacagge tagtagagat ggggttccac tgtgttgccc 4390 agotgetoto gasotoctga cotosegtga tootoocaco toggootooc asagtgotge 4440 gattacaggt gtaagtcact gcgcccagct gtatttttat tttttgagac agggtctcac 4500 totgtcaccc aggeoggatt acagtggcac aaccatgget cactgoagec tegacoaccc 4560 caggotesas egatoctece atotesgict cocaagtace iggggetaca ggggtgtget 4820 accessoring getamatitt gratititing tagagacage gittercoag gittertage 4740 ctgototosa acttgeteto sagtastoca comportoso coccacasag tectggatt acaggogtga gocactgogo etggoettga tetetaettt tatetteetg ettecsagga 4800 satattttt tottotgaat tatoaggoat ttaototigt sattottagt otocotaogo 4860 tigittocgo ocateagasa atggggassa tgattoctac atcaceggge tgtotgagge ctasatgaga togtgtatgt gaaagtgato tgcasacocc acaocstgoc aeggtsaggg 4980 agglagittt tiatiticct gocasagget agcagaaget glacetetig telgagitet 5040 gtotettego tigacaccet teagaggast tootgoctot tecatgggte aggaagaggt 5100 5160 gostagitag titottotgg gigoogagit auttocttoc caccaagigg gittaagccc tageaggest stottssago casestscas tageosttts steotsagos tageootesa 5220 mottogtgtg tggtcagatt tatgitcoat gcgtggggat gtgcaocgga coaggiatgt 5280 stategates acetegates tempetetet etgoteten teteteteca cocttecte 5340 ogtatgacaa goaggotete tetetaggac campaagote tacttetego cagetatete 5460 ectatement excessette titettesse actiectese trecarecat cecacterse totgascact cogagatgag cgagagegee agogggtgte ocgogetgea geoagetttg 6520 catgigotet tettgeteet tegeogitgeg ogtegeggit cogegggatti coggggetet ggretagtet connectena reggianten angoingaco atronatana occascargo 5640 SERTICOCCO ERECOCCOST ESCRESSES ASCESSESC ESSTENSACA ASSANSACE 5700 sagasaagtg gasaagoott tittggggga aascattgat gittgatgit totassaatg 57**8**0 stantgtagt tetcatggga anattagact tgttgggctt masmottitt ottottitec 5820 ctgaagcaga acatgcataa tgttoataaa tattaagcac acanootggo tocattttt ttttttttt gasatagagt ottactgtgt tgocaggotg gagtgcagtg gtgtgatott 5940

[Drawing 4]

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agggggttto aoc	atgttgg co	aggatget	atagetates	tgacctogtg	atcogocago	6120
ottggootoc caa	agtgotg gg	ettacage	ogtgagocac	tgcgcccggc	caacttcacg	6180
tttatacaca coc	atgossa ca	goatoceg	atagagecea	agagocttoo	ctgtacceta	6240
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ostostates cat	tcassa gg	tatgtaga	gaaccaagtg	totocccag	coctgtocto	6360
cagocaccca gtt	teectee et	AFRETANE	ccaccastat	atatttctta	tgtatcccct	6420
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ACTOGRAGET BCA	TOTGAGG CT	TAGTCCCT	GARCTETETG	CCTECCCAGA	CTAGCTGCAC	750 0
CTOCTCATTC CCT	ROGCCCC CT	TECTCTCC	BRAABCCCCC	ABGATGGTGA	Ggteagggco	7620
tgccacccac ggt	agacagg ag	gceagggt	gootggtgoc	CHOEFEROCO	ctcctcactg	7680
coetgectgg god	goodage Te	EGTTTCACC	BABACCTCAG	TROSCTROAT	GCAGAGACCC	7740
TOCTCAAGGG CCG	AGSTGTC CA	COSTAGCT	TOCTOSCTOS	ecccae tcec	AABAACCAGB	7800
QTEACTTCTC BCT	CTCCGTC AC	igtaggtag	gocoocogca	accogggoe	ttttggocac	7860
totottgtgo cat	coaggoc ct	gascosot	cattoctggt	tooccgtggc	agtgetgaet	7920
	agotegratt aca aggregatto aca aggregatto aca aggregatto aca aggregatto aca aggregatto aca aggregato aca attatacas aco acagoacas tatatacas aca acagoacas tatatacas aca acagoacas tatatacas aca acagoacas aca atcagoacas aca acagoacas aca aca aca aca aca aca aca aca aca	agoteggatt acaggogoco acaggeggttto acoatgoteg or contigonaco acaggingto contenta contenta contigonaco acaggingto contenta contigonaco acocococaggingto contenta contenta contigonaco contenta	agotegent acagegoco accacatgo aggegetto accatgoteg gantacage titalacaca cocatgota gantacage titalacaca cocatgota gantacage titalacaca cocatgota gantacage cotatage capantigit cocagingo cototatae cattocaca gantigat gangecacca gittocotoc ciagegrase gitagotac tittocotoc tittgetitag tosgocatge teatacact atocococage tosgocatge tagagacct atocococage tosgocatge gangagecoc appeada tosgogag gongagecoc atocococage tococatgos gococitics acagegrago gotegista gittocococ gangagago gotegista gangatosa agoaniggot groonigag otgantoco gangagago gotegista gangagesa gocattaga goangagagago accitoto titcocitic giasantaga accacage goalgisas goangagago tigocago digantococ gangagago gotegista tatocococ sicagecaga goalgisas gocattaga goangagago tatotagoo tigantococ sicagecaga goalgisas gocattago sicagecaga gangagago cocacage goacococ ticocitic ciagecaga tatotagoo tigantocago tigocago tigoca	agotegent acagegood acceorate congenent aggregation contents occarating granteness of the property of the prop	agotegatt acagegood acceosate congetant tittighti aggegotte acagegood acceosate congetant tittighti aggegotte acceptant acagegood acageatest otogatote tymootogie ottepotod camagigote gestances atagagood tymoocogo tittahaaaa accastood aagaatee atastatet tittahaaagi ootogataa aagaatee atastatee actoasaa gestatee gestatee gettegate tittootogi tittightig geacoacaa sittootod tittightig geacoacaa sittootod tittightig geacoacaa sittootod tittightig geacoacaa sittootod tittightig geacoacaa sittightig geagoacaa tastataact tagigitig geageagaa tagagoocod tagagoaga tagagoocod tagagaaga atagagoocod tagagaaga accastat sittightig geageagaa tagagoocod tagagaagaa atagagaaga accastat sittightig geageagaa atagagoocod aagagagaga accastat sittightig geageagaa atagagaaga accastat tagagagagaa accatotod atagagaagaa atagagaaga accatotaga agagagagaa accatotaga acagagagaa acagagagaa acagaagaa acagagaagaa acagaagaaaaa acagagaaaaaaaa	geotectic asootect tootgetto magtatto octettam octoorage actegrata acagegoed accessate tittitate tittatamag agesetto ocasetate ocasetate tittitate tittatamag agesetto ocasetate ocasetate octoorage ocasetate ocasetate ocasetate ocasetate ocasetate ocasetate octoorage ocotoorage octoorage octoorag

[Drawing 5]

cocceptotet tecettecoc ocaaccocca cactocccat coctetetet ecceacceat 8040 goccatatat goccoccacoc aggacatosa cogatocota contoctaca tetactocta caccegotyr octoacceco tegtyccoty cag6676999 GATCA6676A CCCATATTOS 8100 BATCCAGAAC TCABGGGATT TCTATGACCT STATEGABOB GAGAASTTTG CGACTCTGAC 8160 AGASCISGIS GASTACIACA CICAGCASCA SGITGIOCIS CASGACOSCG ACGGCACCAT CATCCACCTC AASTACCCSC TGAACTSCTC CGATCCCACT ASTGAGASST SARRECTCCS 8280 cacccccgco attoccaago agggatgago oggotoccao cotgaacago cagggaggos 8340 gggagactgg cagcoggogo tgoctacoct coateccete coetecetge accagetggg 8400 gototoasts tocotoctee etsetstoct segacetest stetcasage etsacetace accettices cetasecces agreencese agazanetre eterecetae tecgarance 8520 ctggccgctg caseccaggt cocactggag acagggaggc cactgctggt ggccagcatg 8580 togtecagge cagetetett gttagasage tettetteet etggaatega geetgeette 8640 8700 ctoogtotgo cootcaccoo agoacatgit aggacagiga gyagotgaca otggggigaa gateragate astrotteco sagacactte atmostate coagocecoc ogtaragate 8760 ggtotgtoot gtggggtoas ataggtotoc ggooossacs gagstoattg agagosogst 8820 gtgaegtgtt cecctgtgta augtgtotca cgctgtoccg ggoacagagt autactocag gesttteett eetgiggeet ecoegaciee teetgiggte teecasagge sigggetggg esctserese totseatest cotcateaca contesctor titoagramo escatotosa 9000 tgccagatoc cottagagta sagggcagog gastaacgot agggggtttt cacatgcaco 9060 octgggccas gcogactige octtgeogtg gatooctgca ticatggate ggttattgas strategers acctigated tracegetts caractetet gagatteres ectocasset 9180 gcatcantat tittggtcaa ggcactgatt gaaacttaga gctggatteg gtcacggtgc 9240 ageoetgtgg cooseetggg aggesteett teetggsteg gesteettes aggestteet 9300 totototyty agostoscat ggotggotoc gtgtotgocc cotgocotto otottoccca 9380 9420 cognanced caggingent tighoacoga gaocototas agotoateto ctototitot octigootco agocaggaga ggaggaoggg cigaocagig octggaggig gaagagagga 9480 genggecce aggaggecce tgengaggag getgaggeet gagttenagg agnagagaga 9540 agagagagaa ggaagggagg goagtgoogg ggogggaggt taagacoagg gaagoogoac tegaggood titeggigae cogiccoans agcoarteto accocigage cigggagigi 9660 gigagagect cittotocca agitotgota igicototgo citatotata egectocico 9720 totgogagae titigoatotg tooctoggig gototgogot tootgiggio agootgecat 9780 ttgcatggag acttoctcat cotggggeet gagggaaggg getoagococ otcocogeta 9900 cotggggtoc tagootgtoc coaggoggtg ggotgaagta goccagtegg gttaggaggo

[Drawing 6]

totegregato totoggotge agtoscotoo gegonggest gagategatt gegacagact 9960 ggtoctoccc tocttoccc catooctgog gttggaaast ttgcccgccc toccctogtc 10020 octgegotga ggasacotca casoctcact totoactoto tooccagaag gagttttgtg ttttttccat cacgtggttt octgtggggo tgggotttgt ggggctacag tttcctcctg 10140 greesgret gigetteggs gasagggett agittotsett totgoocise cagoccette 10200 asstcogitt gescootggg otcocottos gigacetest coagggesco coagsaccec 10260 ctecapeact ctttecceag tagggttgte tteccegect ccctggcgga gogoacocca 10320 teagestics tigigacity agtotytyty tooststoos sooseteest giggiginge 10380 ctoggtotge gtttetettt gootetggte totgetgggg eacagtecea teetteacgg 10440 agattostoc ttagettete tectecasat attitgaata tigeoageet ttetgeettt 10500 cagaggingg cicinggite gaageceggi tagaactety gaggetagga tggetigaac cterrarete esercteras assectatas occupante continues otrespance 10620 gagototgga agottgocot agagtoagto aagggooota ggocagtgag teacagotca 10680 10740 gostoastit octoatotat easatgggg taatatosta octagototo agoststits tragaracet eastraggts stagetting associates orcastreet speakerst 10800 aggigatiga titooggood otototetes etgictotes teagogoott occorteteso 10860 otgggtotta cottocctga ogotgoctto totagGTBGT ACCATGGCCA CATGTCTGGC 10920 GESCASSICAS ASACSICTECT GCASGCCAAG SSCSAGCCCT GGACGTTTCT TGTGCGTGAG 10980 ASCETCAGOD AGOCTOGASA CITOGTECTT TOTETOCTCA GTEACGAGOD GAAGGOTGGO 11040 COAGGCTCCC CGCTCAGGGT CACCCACATC AAGGTCATGT GCGAGgtagg goaggggggg 11100 ggogggggag octotgotga ggotcotgto tgtgaccaca gtgtgggtag cagggagggt 11160 ctgootgggc ttgasttcse ggctggggac ccagggaggg agactcaagt cotgtgaatg 11220 goctanttig gotococca gGGTGGACGC TACACAGTGG GTGGTTTGGA GACCTTCGAC 11280 ASCOTCACOS ACCTEGTEGA COATTTOAAG AAGACOGGGA TTBAGGAGGC CTCABGCGCC 11340 ITTETCTACC TGCGGCAGgt caggggtggg cocagotgcc tecceactte coctgagetg 11400 tecceoagat gigagettet gggatototg agitgetgae tictegetet tecceacece 11460 AUCCOTACTA TECCACRAGE GTGAATECES CTEACATTGA GAACCBAGTS TTBOAACTBA 11520 ACAAGAAGCA GGAGTCCGAG GATACAGCCA AGGCTGGCTT CTGGGAGGAG TTTGAGUTGC 11580 sterteres cogecarge treescapt sageteres cascaccte resoccoage 11640 eggacacett ecceteettg cocacetetg etcetgacce accessegtg agetecces 11700 atgratgece tettigggag otgatgetes ittoccomec cacateteng AGITTGCAGA 11760 ASCARBAGGT GAARAACTTG CACCAGGGTC TEGAAGGECA GOBECCAGAG AACAAGGGCA 11820 AGAACCECTA CAAGAACATT CTCCCCTgtg agcaccoagg otgocccatt cacccaggat 11880

[Drawing 7]

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                                                                      12420
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genetacete totocaceot tecetocaea ACCASCTSCT ASSCCCTBAT GASAACECTA
                                                                      12540
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                                                                      12660
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                                                                      13200
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                                                                      13380
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                                                                      13560
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                                                                      13620
 ageocatoeg tecatecane asatgittigg geoggtgees ggeneteaga sestagages
                                                                      13680
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CCBEAACAAA TGCGTCCCAT ACTOGCCCGA GGTGGGCATG CAGCGTGCTT ATGCGCCCTA
                                                                      13800
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[Drawing 8]

CTCCCCCCTG GACAATgtgs gtggocccca cgcootgocc cattcoggga gtocotocot 13920 gractigate tectetotes temperature transferst resultation garageses 13980 ERRECACTES COCTATETCC TORRESTARG GAGACCTGAT TOGGGAGATC TGGCATTACC 14040 AGTACCTGAS CTROCCCGAC CATEGOGITCS CCARTGASCS TORRESTETS CTCARCTTCS 14100 TEGRICAGAT CAACCAGOGG CAGGAAAGTC TECCTCACGC AGEGCCCATC ATOSTECACT 14220 GCAGgtgagg atgatastcc tgatggtagt agtgacagct gagaagtana tactgotaag trocatrare tritateare satatearer timestores cattrartes estocates 14280 eccocgott ctootgggto coctcatgge tocagaacce tgggtggato gtggotggaa 14340 ecagococae titiggecete tgectgtggg tatetteete agageeetet coggatgtae 14400 catotogoco ascoctgoca satscagage aggagocogg gaccoagtty ctggocaggo 14450 ocsagotagt cagggcagg cogggcagge accescagta ggcctgtgtc coggctgctc 14520 egettietet egaggteeca tietgitggi tietteteec aggaacatet atgaggostg 14580 tgotcoccat tectectett tttecatogg tageogragg getteggett ettectgaet 14640 ctgoootete tooosgette cocaggeagt goccesteet ggoccecagg getstatung 14700 gateggigat gottottigg ggotgoacat aactostete totatotaco egcatettig 14760 14820 tgatcaggag acototggts aggtgcagag gtgggggctg caaggaggag caggggttcc acaggigago coacigagot ggociggoti gggiggatga gaggoagigg gigcagggoc 14940 octockotta coaketetet aktettekae santtaetta aettitotaa coetoakott cotostotat assetcegge totosgggtt gtogtgegge ctosetgage coctatogtt. 15000 gtggotggaa ttoogtoago cotcaessec tgggogetgt tectegttte gtaectoeca 15060 toaggoagag aataggggas tgggsaootg oottgooogg gtocottooc actooctoog 15120 tresoccese gootgorsog goototggot toctcotett occccagcag ctgittgtcc 15180 tgggacaggg caagtoggot gaatotagag gtgooocoga tgggotgtoo ggggacgcgg 15240 ctotatcota tactototca gagacaggoo catoocogag agotacceto etgotoacoc 15300 15360 gocacacaca cattcacaca cttottgasa goccostggo ctttatttag acgttacage saggageter stategeres tigitities castoteret tiganatias acasegeresc 15420 teagggeste agettgetgg geteagetga gggtgggeet ggggtetece tgaggtetgt 15480 ttgcccageg ctgggaaagg agageaactt cotactgcac tgotcocctg agtcocctga 15540 coctgtgccc cogcaccety ctgtctcage gctatoottt coctgaogto agggtttgaa 15600 sessasses astrasecca tecteseses cectroates otocttoses escapences 15660 gaggeteag ggtacetggg ageoggeagg acagtggtgg gatttggggg teccaggtet 15720 tocessetse sescasocac toactaggas tsassastos sososassas tssassasses 15780 saggatggtg geagetgggg agoosgogte agosoogong agooogangt ggagegtgte

[Drawing 9]

15900 categoagage terrecasace togateates etteccerri gasocterge acattocoto coatcactgg aggetcagge tgetcetgtg gtgectgggg etggagetga gegetgggta 15060 occccctice ogggegege tigacteroc totgateres occccgtett tecccag080 16020 CRECATORRO COCACARGOA COATCATTRT CATOGACATR CTCATGGAGA ACATCTOCAC 16080 CAMBRITISE ERECOCKER ERECTER ERECTER REPORT OF THE PROPERTY OF 16140 ctatgeetgg acetgaggtt tgactgeece ceacocagGC CTGGACTGT6 ACATTGACAT 16200 CCAGAAGACC ATCCAGATGG TECCOGCGCA GCGCTCGGGC ATGGTGCAGA CGGAGGCGCA 16260 GTACAABITC ATCTACBTGG CCATCGCCCA BITCATTBAA ACCACTAAGA AGAAGCTGGA 16320 GETOCTECAS stgostscas ascasssoct sessesses sessetscas tecassates 16380 16440 gtgeomeetg geoetgetgg gaecaccacc tteccactgt ecetotgece acagTC6CAG AAGGGCCAGG AGTCGGAGTA CGGGAAGATC ACCTATCCCC CAGCCATGAA GAATGCCCAT 16500 SCCAAGGCCT CCCGCACCTC GTCCAAgtga gtegcoctga ctgocactgo ccggcatoca 16560 coccttigto otgoccagoo ogatoctoac titotggaga ggacaagtgi tgoagotggg 16620 gggacotggo ticaagitos sgotiggito tomococito igitosiasg catitooiga 18890 gigocoacec gigigggeet oigotaggia coagoagoge actogigiat gagatgiage 16740 16800 ctetricete taggagette regictagie carrescert gretgogies cotetrages sergiaces seggeracts coastgoogs stoccoctst sotstotect secotspace 16860 16920 anotgootgt nottgoocco otgoscoogg otgosgACAC AASGAGGATG TGTATGAGAA CCTGCACACT AAGAACAAGA BGGAGGAGAA AGTGAAGAAG CAGCGGTCAG CAGACAAGGA 18980 SAASASCAAS SCITCCCTCA AGAGGAAGTS AGCSSTGCTG TCCTCAGGTG GCCATGctsc 17040 agetettetg cetgggtgte etcectgooc tgeoctgtgt cottggetce actgcettee 17100 otgggtggat ggggtggoog cagootoatt otgtgottoo oagotgooco agacoctott 17160 gittocaccic caggittocag ciaccototo actocotoac tocottotot tggcagCCTC 17220 ABCOCTEACE CTETEGRAGE ATTTCCCEAT GRACARACTC ACAACCTEAA CCTARGASTE 17280 ODCCATTCTT FTGTAATTTA AATGCCTGCA TCCCCCCCCC CTCTCCCTGA CCCTGTATAT 17340 AECOCAGOCA GEOCOCAGGO AGGEOCAADO OTTOTOCTOT TETAAATAAA GOCCTEGRAT 17400 17460 CACTetetet egectotese coettigett geocagtese temmegeca eseggesegg 17520 cappatement aactetetet gootoogteo gtroctogog transactoo goottoogto agacegacet gggtcgggac tocgcotogo acgtgggagg gtgaccgtgg gtgaagetco 17580 coagtotoot totttaasat ggaggggat cataacaggg tggttgtgas asgcaccgag 17640 atgacggotg acgatasgac gggcacagtg actostosca ogcttgccat gtgcccaggc 17700 17760 actasasage tacacacett agticagiot aggoactict gicattotes tittacegig gorganacty aggracages saucteagts acttrytose ttgoccaagy teacagget 17820

[Drawing 10]

stggsscagt gaggetgg	e ttogaacoca	geotetotga	ococagagoo	oscactoctt	17880
socotggagt tgcagctg	g gccaccctca	REEEEEcoot	gatoscaoto	ooctgetget	17940
gagttocaga totgaacte	a gaagagtagt	taacagoogg	eagogcagac	ctgaggccag	18000
occepted tocotets	g cgggaacagg	gacaggetee	teagageace	cgggcacgoo	18060
cagatactes octoates	g googetgetg	cocttatoot	ottgggoaga	gtttgaagag	18120
otggotgacg tgaagagt	c tttgtttttt	gtoocotctt	ecttoccoca	tgtoaggagt	18180
ggggtttett etttettt	a sacactggtg	toctgeggag	tamagooggt	gggagtcatc	18240
octcaggaag tgctggcgc					18300
agaggotggg cgtgoatti					18360
acegtococc gacetecti					18404

[Drawing 11]

totocotgac	cagootoota	agttoongge	EFTCEFFFF	otgtgaatgo	otgtgacgco	60
sessetts	tanggatttg	ctgagtestg	caogoocago	ototgegett	teggacctgt	120
sctstctcas	ectttccage	arterrorco	agcacttoot	gagggatgac	teccacegge	180
tttactocco	aggadaocag	igittomaat	acagaageaa	occoectoct	gacatggggg	240
anggragagg	ggaceaseaa	casagcacto	ttcacgtcag	ccagototto	assototgoo	300
ceageggata	agggoagoag	cggoctggat	gagggggggga	gotgggogtg	occeggetgot	360
ctgaggagoc	tgtooctgtt	cocyccagag	EEEacEcaEc	ogggotggoo	toaggtotgo	420
gottooggot	gttanotact	ottottagtt	cagatotgga	actcagoato	aggggagtgt	480
gatoagggco	cocctgaggg	tggcocoago	tgoaacteca	gggtaeggeg	tgtgggetet	540
ggggtoagac	agootgggtt	cgastooceg	cotcactgtt	contagooot	gtgaocttgg	600
gcaagtgacc	aagttectta	gtittiotgt	coctoagttt	ocgocacggt.	asastgagaa	660
tgaoagaagt	goctagaotg	aecteacgtg	tgtegtettt	tagtgootgg	gcacatggca	720
agogtgtgat	gagtosotgt	gocogtotta	togtoagoog	toatotoggt	gottttcaca	780
acceccotgt	tatgatogoc	ctccatttta	aagaaggaga	ctggggaget	teacceaegg	840
tosocctocc	acgtgcgagg	oggagtooog	acconcetoo	gtotgacgga	aggoggagot	900
ttcacgogag	goacgcaogg	aggonoacac	agtteoccat	ootgoootgo	octotggoog	960
occactoact	gggcaagoaa	agggotoage	ggogooocc	AGTGATCCCA	GGGCTTTATT	1020
TACAAGAGGA	BAAGOSTTOS	CCCTGCCTGG	GOCCTGGCTG	BOCTATATAC	AGGGTCAGGG	1080
AGAGGTGGGG	GCGATGCAGC	CATTTAAATT	AGAAAAGAAT	BOOGCACTCC	TARGTTCARG	1140
TTETGAGTCT	STCCATCGCS	AAATGCTTCC	ACASSATCAS	GGCTGAGG ct	gccaagagaa	1200
egagteagg	gagtgagagg	gtagctagas	coteeneete	gaacaagagg	gtotggggga	1260
gotgegasgo	acagaatgag	getgeggees	coccatcoac	CORREGUEE	cagtggagcc	1320
aaggacacag	ggcagggcag	EESEESCHOO	CHECAGAR	agotgtacCA	TOGCCACCTG	1390
AGGACAGCAC	CECTCACTTC	CTCTTGAGGG	AACCCTTECT	сттетестта	TOTGCTGACC	1440
OCTOCTTCTT	CACTITOTOC	TOCCTOTTET	TOTTARTETS	CAGGTTCTCA	TACACATCCT	1500
CCTTGTGTct	geageegggt	ECSESSESS	aagtacaggo	agttggtgca	ggtcaggaga	1560
cagcacaggg	ggeoccggca	ctggcagtcc	cetetggeen	occeptetos	caggigacgo	1620
agocacggto	cctgcactag	actocaaget	cctagaggac	agegectaca	totoatacac	1680
gagtgogetg	etggtaccta	gcagaggocc	acacgtgtgg	gcactcagga	satgettatg	1740
accagaaggg	gtgagaacca	agcotgaact	tgaagccegg	tececeage	tgcsscactt	1800
gtoctotoce	geongtgagg	atogggetgg	gonggacen	agggtggetg	oogggongtg	1860
contonegg	ocactoacTT	GGACGAGGTG	CGGGAGGOCT	TEECATEGGC	ATTOTTCATE	1920
OCTO0606AT	AGGTGATGTT	COCRTACTOC	GACTOCTEGO	COTTOTGOGA	ctgtgggcag	1980

[Drawing 12]

2040 agggacagtg ggaaggtggt ggtoocagoa gggccaggtg gcacccatcc tgcactgoag occoccoco occoaggoco tgototgoac goacCTGCA6 GACCTCCA6C TTCTTCTTAG TESTITICAAT GAACTESSEG ATSSCCACST ASATSAACTT STACTSCSCC TCCSTCTSCA 2160 CCATGCCCBA GCGCTGCGCC CGCACCATCT BGATGGTCTT CTGGATGTCA ATRICACAGT 2220 CCAGGCotgg gtgggggga gtossacotc aggtocagge ataggoggac acogaggggc 2280 tgotoaccoc ocaoococaa accoccaggt goocotcacC CTTGGTGGAG ATGTTCTCCA 2340 TRASCATOTO GATGACAATG ATGGTGCCTG TGCGGCCGAT GCCGGCGctg gggasagoog 2400 ggggtgocat cagaggoosg toasgoootc cocgggaagg ggggtaccca gogotcagot 2460 ocagcoocag gosooscagg agosgoctga goetccagtg atgggaggga atgtguccag 2520 ggtoacoggg casgigatga tggaggitte cocagotote catggacace etocacotog 2580 ggetetgegg tgetgaeget ggeteeceag etgeogecat cottenetee tengetoete 2640 2700 gegeogacte eteactecta gtgagtgget geococaoce eggaagacet gggaececon astoccacca ctgtoctgoc ggotoccagg taccotgage cotcoccage totocctgan 2760 sensitiates ascetototo asontssott cantingett theetteam coctemente 2820 agggasagga tagocotgag acagoagggt gogggggoac agggtoaggg gactcagggg 2880 agoagtgoag taggaagttt ototoottto coagoootgg goaanoagac otoagggaga 2940 ccccaggccc accotcaget gagcocages agotgatgcc etgagtegog etgtetaatt 3000 tossagges attetosses stasgoogge soaccoactt cottectate acetetaset. 3060 eauggocatg aggetttoss gasgigigig satgigigis teauggagige geagengegi 3120 agototoggg gatgggcoty tocotgagag agoacaggac agagocgogt ococggacag 3180 occurrence acceptate attrageous ettercetet cocagescas acasetrote 3240 expressing agrangeous aggregates aggregage tooleggage saginguals. 3300 ggaceggggc aaggeaggtt cocattecoc tattotetge etgatgtgag ttactasact 3360 egtaecagog cocagititit gagggotgac ggasticcag coacaacgat agggicteat 3420 tgagttotca ogacasooct gagatoctga tittacagat racgaagote agggttagas 3480 magitaagta attigiccas gaccacacag ciggiaagcg gaggggcoot gcacccactg 3540 octotoatco accoaggoca ggocagotoa gtgggotoac otgtggaaco cotgetecte 3600 ottecasco conoctoteo acottacone agriculte atcacasco tecesetara 3660 tagacagagg agitatgigo agococasag aagostosoo ostococaca cagocotggg 3720 Excontents atteactace taxtanget attages and attages and attages and attages at 3780 sagocotgog gotacogatg gassaagagg aggastgggg agosostgoo tostagetgt тестругара адаасскае адаатрукае сторинава адопракову содужения 3900 ggeotactgt gggtgcctgc coggeottgc cotgactage ttgggoctgg coagcasetg 3960

[Drawing 13]

ggtocoggge testestetg tattiggeng ggttgggoga gatggtacet coggagaggg 4080 ptotragges estacoceos recessageo camerterre otrattoras ocacestora occaggatto tggagocetg agggacoca ggagaagoog ggggtgagog gagggoacto 4140 satgigogag ctasogitta tattgottat ascagotost ggoacitago agistitaci 4200 totoagotat cactactace atcaggatta toatectose CTGCAGTGCA CGATGATGGG 4260 COCTROSTEA GROAGACTIT COTROCOCTE STYGATOTEG TODARGARO TEARGACACO 4320 CCCAGGCTCA CTGGGGACCC CATGGTCGGG CCAGGCTCAGG TACTGGTAAT GCCAGATCFC 4380 CCGAATCAGG TCTCCctaag oogaggacat agggtcagtg ooccotooto totoggaaca octostocat otcaccotac cosaccaras assessosa stocassuse seactocoss 4500 setggggong ggogtggggg conctoncAT TGTCCAGCGG GGAGACCTGT AAGGTACGGA 4580 ETTTETATTC GETTETCTCA TECTCCCCCC ACTTGETCAC ASASTASSOC CCATAASCAC 4620 SCISCATGCC CACCICGGGC CASTATGGGA CSCATTIGIT otggstaggg agggtogggt 4680 ggggatgagg cacagagcag ggoactgtgg cocatocoag gtoctgotot atgttetgag 4740 tgcctggcac cggcccaaac atttgttgga tggacggatg ggctggcaga gaggcaggag 4900 acctgracte togictoaga tosaccacca acagatanty agreetance agecactica 4920 cognitions of the contract to a constant the constant and the contract to a constant the constant to a tronscore acquettese arecacanat ascenceagt etgigococt gigootogag 4980 5040 featocourant statements contantes coccionate standates criticosans ogtaattoto cotctaggto otggaggaaa caaggootgg attogcagag gaggoagego 6100 tooggotgig tottomeane ogaggoatot goonggonga gategiging agagagtoat 5160 ttoctagoag assatgaget tgtgessaga saggetggga acetgasega getggggetg 5220 ctggggggg tgagcottoe agageagogt gcgggtoega gggaagocac cagacettga strocargoe tragagises actitatece assertesce arrageests gasgagatit 5340 cagasaggas ggaectigac coagitigis teacggaige galeactica atggoagcac 5400 agagggtgot gigtaggggg agootgoagg gtaaggggca gactocagag agatotatgg 5460 SECRETARE SCREENING COLUMN SECRETARE SCREENING PAREOREME 5520 5580 ESCRECTER ASCOTERATO TERRECTOR SAMPRECCOR SESTECATEC ASSESSECTES gggtcacgtt gtgtagtega gacttigtea ctageoctea gttteecaac sagggoogga RAAN occidences tgagatecet ttotgecotg gestetgact getettegae ttastggest 5700 geocecases acgurgaces strossures generatore octaccosoc CTTTCTCCAC 5760 CTCTCGGGTG GTCATGACGA TGACACGGCT GTTCTCCTGC CACGCCATCT GCCAGAAGTC 5820 ATTEACORTS GOCTOCAGAD AACOCTORCT GOORATRIAG STCTTAROST TOTCATCAGE FRAN SCCTAGCAGC TEGITOTERS ognasserts geragaeges occceagaes strascocct 5940

[Drawing 14]

tggoccagca	caggoodtga	accaetgoca	cootoacooa	tgoaagaago	oegottoggo	6000
tttgcccago	tgtctctgga	totegggtoo	coocsocsga	cegggaetto	0002EEC000	6060
goototooto	ccacgtggcc	cacactgotg	acCTTGATGT	AGTTG6CATT	GATGTAGTCG	6120
BACCCGGGGA	TETTACTETC	COSTCOCTEC	AGGATCACTC	GGCTGTBGTC	AActgggagt	6180
gggoggagag	gaggogagat	ggtctgggtt	agccagggac	toaccacacc	tgggtcagac	6240
octoctgago	taecaggoca	ttotggtoto	agaagccatg	aggettgogg	gggactgtgg	6300
gongongggg	tgggggatca	totgtetgge	greagtgage	gegttosons	ggaggttggg	6360
agcagggggc	nacctgootg	ctegtggtgg	ggaggoaott	ctgggggggg	tagaggoott	6420
ttggagaagg	Eggaggetet	steesessts	tggggagatg	gococtoctg	gogtegaggg	6480
tggagacctg	tgegatgagg	seasscasct	ER CEONETER	centatoots	ggtgeatggg	6540
goagoctggg	tgctcscA60	GGAGAATGTT	CT TET ABOGG	TTCTTGCCCT	TETTCTCTGG	6600
CONCTRACCCT	TOCAGACGCT	GGTBCAAGTT	OT TOACOTOC	TECTTCTBCA	AACTotgaga	6660
tetesetese	gaaatgagce	toagotooca	aagagggcat	ocatcggggg	agotoacgtg	6720
gggtgggtoa	ggagcagagg	tgggcaagga	es essecute	teegeetggg	gococaggoo	6780
gotgocacca	octoagotgo	coongcootg	ocegtoocca	ccatgcacCT	CAAACTCCTC	6840
CCAGAAGCCA	GCCTTGGCTG	TATOCTOSGA	CTCCTOCTTC	TTOTTCAGTT	CCAACACTCG	6900
GTTCTCAATG	TCAGCCGCAT	TCACCCTCGT	OGCATASTAC	0Gctggggtg	gggaggagog	6960
egangteage	aactcagaga	teccagaage	tescatetes	eggacagoto	agggeagtg	7020
EREMERCARC	taggeccacc	cctgacCTQC	CECABBTAGA	CAAAGGCGCC	TGAGGCCTCC	7080
TCAATCCCCC	TOTTOTTGAA	ATGCTCCACC	AGGTCCETGA	BOCTOTOBAA	GGTCTCCAAA	7140
CCACCCACTG	TETAGOGTOC	ACCOTEREE	gagocasatt	aggooattos	caggacttga	720Ó
gtotocotco	ctgggtcccc	agocttguat	toangooong	geagecocte	ootgocaoco	7260
acactgtggt	cecagacagg	agootcagoa	gaggetocco	ogcogcetag	ctgccttacC	7320
TOBCACATGA	CCTTGATGTG	GGTGACCCTG	AGCGGGGAGC	CTGGGCCAGC	CTTGGGCTGG	7380
TCACTGAGCA	CABAAAGCAC	GAAGTCTCCA	GGCTGGCTGA	GCCTCTCACG	CACAAGAAAC	7440
GTCCAGGSCT	COCCCTTGGC	CTGCAGCAGC	GTCTCTGCCT	GCCCGCCAGA	CATGTGGCGA	7500
TG6TACCACc	tagagaaggo	agogtoaggg	aaggtaagac	ocaggocaca	EEEEnaggog	7560
ctgagcagag	acattcacag	agaggggoog	gasatoaago	acctactgtg	tgccaggcac	7620
tecectacat	gettecasat	ccaccacctc	atttaggtot	otesessees	tgctgagage	7680
taggtatgat	attaccccca	ttttataget	gaggasactg	acgotyaget	gttactcact	7740
FECCHBEFEC	cottgaotga	ototegggoa	egottcoage	gctotgttgc	ccaggetgga	7800
gtgcegtggc	goggttacag	ctototgoag	octogancto	ocaggiticas	gocatocteg	7860
cctccagagt	totanooggg	cttcgaaooc	ag agcooacc	totgesaggo	agaanggetg	7920

[Drawing 15]

goastattoe asstatting agragament classrates atctocetes aresterrac 7980 tgtgooccag cagagaccag aggcasagag aaacgcagac cgaggcaca coacagggag 8040 testessaga tesacaca sautosagto acamerang cesatesset sesetecese 8100 aggraggogg ggangacaac occacteggg amagagtggt gtagggggtt otggggtgoo 8160 otgestasta toschassa gragoccare ettosasore atttesagge gotetosger 8220 cegasagoag sactaagooc tttoccogsa goscoccoot ttoccaggag gasactgteg 8280 coccacang cocagoccca cagganacca ogtgatggan assecaceas ectocttotg regagagagt ragaagtgag stigigaggt tiootoagco oagggaogag gegaggggg 8400 gcaaattttc osacogoagg gatgggggga aggaggggag gacoagtctg toccanocca 8460 totoaccoot gooograggt gastooague gagagaouce cagagestee taaccecact 8520 segetactic agoocaccgo otggggacag gotaggacco caggtagogs ggaggggget gagococtto cotcagnoco cangatgang sagtotocat gosestatos ggotgacoso 8640 aggangogos gagooscoga gggacagatg canattotog cagaggagga ggogoscaga 8700 cesggcageg geoecescag escotgggsg sampageote tesceneste conggetong gggtgacact ggctcctggg acgggtcacc camaggggc tocagtgogg ottocotggt 8820 ottamoetec egeocogges etgecetece tteettetet etottetete ttetoettgs 8880 accoaggest cagostests tgcagggge testgggges etgetestet ettecacete ROAD caggosctge toagocogto otcototoot ggotggaggo saggagasag agaggacatg agotttagag ggtotoggtg ocassagooc ootgagtgtt goggtgegga agaggaaggs 9060 cagggggcag acacggagco agcostgtga ggotosoaga gagagggaag goottgaagg 9120 aggocrator aggaeaggag gortocoagg tgggocacag ggctgcaccg tgaccgaate 9180 cagototaag titosatoag tgootigaoo aassatatig atgoagtitg gaggoogsa 9240 totoagagag ectgoaaget eggagagea agettoccea toutttoaat aaccestoca 9300 tgestgongg getccacgge engggenegt eggettegeo cargagagen tgtganneo 9380 occtagogtt attoogctge cotttactet aaggggatet ggcattgaga tgeggetget 9420 gamagagec atggtgtest gaggagentt cagageccec agoccocage coatgoottt 9480 gggagaccac aggaggagte ggggaggecca cagggagggag atgootggag tattactots 9540 tgocoggac agogtgagac actttaceca ggtgaecact toacatogtg ctotoaatga 9800 tototette georgagae etattigace concaggaca gacccatcoc caeggggegg ctegescase gostosagie tottegosag cattosicos catoticaco coagistose 9720 ctectosoty tectasesty tyctgregty aggregospac ggaggaagge aggetegatt 9780 congegues aegegettic teacescage getggcetge acgecatget ggocaccage 9840 agtggootoc otgtotocag tggggcotgg gttgcagcgg coagggotcc cggagtaggg

[Drawing 16]

ogaggosgot ttotgtggot tootoggggt taggtggasa gggtggtagg ttaggototg 9960 agnoaceagg teccaggaca geagggagga gggacattga gagcoorage tggtgeaggg agggengegg atggagggte ggoagoggoog gotgooagto tooctgooto cotggotstt 10080 cagggtggga googgoteat coctgettgg gaatggoggg ggtgoggago cotcaeCTCT 10140 CACTACTEGG ATCGGAGCAB TTCAGCGGGT ACTTGAGGTG GATGATGGTG CCGTCGCGGT 10200 CCTBCAGGAC ACCCTGCTGC TGAGTGTAGT ACTCCACGAG CTCTGTCAGA GTCGCAAACT 10260 TOTOCCOTOC ATACAGOTGA TAGAAATOCO CTGAGTTOTG GATCOGAATA TREGTCACCT 10320 GATCCCCCAC Cotgoogggo acceggoggt gaggooagtc ggtgcaggag tagaggoagg 10380 agggcaggga toggctgagg tootgggtgg gggcacacat gggcatgggt gggcacagac 10440 aggestaggs agtstaggag ttaggagosa gagescegac gaggagtosa cectaccaca 10500 gggaecoagg satgagtggt toagggcotg gatggcecas gagagtggcc sasatgcccg 10560 ERETTECHE ERECCOROCT ACCTGACEGA GAGCGAGAAG TCACCCTGGT TCTTGCGACT 10620 EGGCCGASCC AGGAAGCTAC CGTGGACACC TORGCCCTTG AGCAGGGTCT CTGGATCCAG 10680 COCACTGAGG TCTCGGTGAA ACCACCTEER oggoccaggo agggoagtga ggaggggtoo 10740 ogigggeace aggeacecti geeteetgie taecgigggi ggeaggeet taeCTCACCA TARAN TOCTOGOGGO TTCCGGRAGAG GAAGGGGCC CAGGGRAATGA GGAGGTGCAG CTAGTCTGGG 10860 CARRICAGAGA GCTCARRIGAC TAARCCTCAG ATRICARCTCC CARTRODGGG CORCCTCACC 10920 ACCCCCGGTG GTCCCAGTTC TGGGGCTGCC ACTCCACTgg ootggggcag ocggcagggc 10980 EEFESCARGA ARAGROGOS OCCACCOCT GEGEGRACTO ACTIGICAT CTOSTITUS 11040 aponcagona gygangagan gongagongg gtggonotgg gygtggggac gyggoccact 11100 11160 toccapacet circustosa exectosuma gostagosti scapatatet gacascacat graptanga cacatgigig cottgonous toonggoods gatastacti cacguatgog 11220 saccongacy giocoacygo etcictocot octobaccay oigotittae goagocotoc 11280 11340 otgęcocace otastascaa tgategoggt gacaacteto atogagtgag tootgotgga teocesces tetestataa teesettose atesettete tentteettt steasonaas 11400 11460 coctatgaes caggagetat ettiateatt tiacagacas ggascetgag getcaggag tttaaggost tgootgagto catcagtgag toagetgoag tgoonggatt canaecoaga 11520 cagtocgett coasagocae tectttesac tootetacac cagocotose egottossae 11580 ascagagatt asgagootoo tigicossag gegotgoost gggagoagga gaotaatagi 11640 tangeoutty consequity cangagagas gggtgccctc ctctmaccan catatgoagt 11700 tigiccatgo cacigiggoa gagggaigga caacaigaco cigagcagco cigicaacai 11760 gggtcottto tasttottgt cootaggcet ctgccctgcc ccagcactoc caccaggggg 11820 octatotoct gegggotgeg ggacanggto ctcaccatge ctgattttot canggetget 11880

[Drawing 17]

tgatettaga	ggocctmaac	actaaggtat	atgatgotgo	ctacctgtaa	ttocasatac	11940
asscatosso	acceccesac	cassacgagg	assagcaget	ceacagegga	tacateagae	12000
acacatattg	gtggetteec	ctagggaggg	asactgggtg	gotgengenc	agggotgggg	12060
gagacacttg	gttototaca	taootttttg	estettetat	gatgggoatg	cattaccttt	12120
atassastas	atatectesc	tgggaacaat	ttotgggass	cttttagggt	acetetanes.	12180
ctctttgtct	ctatctggat	gotgtttgca	tegeteteta	toanceteau	gttggcoggg	12240
cgcagtggct	cacgoctgta	atcocagcac	tttgggaggo	caaggotggo	ggatoaogag	12300
gtcaggagat	ogagacoato	ctggcossos	tggtgaaecc	occtototec	tassastaca	12380
eaceacttag	etgggcatgg	tggtgggggg	otgteetocc	agctactogg	gaggttgaag	12420
caggagaato	acttgaaoco	aggaagooga	ggittgoagog	agocangato	aceccactge	12480
actcoagoot	ggoasoacag	taggactota	tttcssesses	888888888	tggagcoagg	12540
ttgtgtgott	aatatttatg	ascattatge	atgttetget	tcaggtanaa	Representa	12600
ttitaagocc	aacsagtote	attttcccat	gataactaca	ttatcatttt	tagaaacatc	12660
asscatcast	gitticcccc	assasagget	tttccacttt	tetttettte	tttttgttot	12720
atttgtttct	ggttttcttc	teteactggt	gcoctggggs	atotgootgg	tegetttett	12780
gostggtoca	gotttgacta	coocttoago	ttegacacca	coccagagoo	ooggaaatoo	12840
goggsaccec	cacgoccado	gocaaggago	aagaagagce	catgosasgo	tggctgcago	12900
gogggacaco	egetggeget	ctegeteate	toggegtgtt	ozgegtoczg	tgogatgoot	12960
ggceotgago	aagtgttcaa	caascaagot	ogtoagtoos	tagcoacata	cetggecaca	13020
agtacagett	cotggtoota	cacacago	ctgottgtoa	taegoaggoa	agggtgcaca	13080
ozcagtosos	gcacecacac	ctcatcatoo	atgtocacct	acacatac	ctggtccggt	13140
gcaoatoooc	acgostggas	catacatots	accecacacg	augtittegg	tocatgotos	13200
goaccaaacg	oocaotgoac	octggooosa	agacactoct	tocagggett	assoccactt	13260
eeteegaagg	aattaaotog	goaccoagsa	gaeacteact	aggoacotot	teetgaeeca	13320
tggaagaggo	aggeattoot	ctgaegggtg	tomagecom	agacagaact	cagacaagag	13380
gtecagette	tgetateett	tggcaggaas	etassasect	acctccctta	cottagoate	13440
stetessett	tgcagetcec	tttcacatec	acgatetoat	ttaggoctos	geoagocotg	13500
tgatgtagga	atcattitcc	ccattttctt	atgggoggan	aceagogtag	ggagecteag	13560
aattacaaga	gteastgcct	gateattosg	anga naznan	tatttccttg	gaagcaggaa	13620
getasaagte	gagatcaagg	ccaggegeag	tegetcaege	ctgtaatccc	agcactttgt	13680
seeset sage	ctggtggatt	acttgacacc	angittgage	geagectagg	asacctggag	13740
assocctato	totacaaaaa	atacaaaatt	tegocaggtg	tggtagoaca	cocctgtage	13800
cccapptact	teggagactg	agutgggagg	atogettgag	octgesetes	togaggotgo	13860

[Drawing 18]

estragocat artistacca cigiaatcor socientes carestrana contrictos 13920 essentases stategotus accoestase that accoust autocoasta ottissesse 13990 oogaggiggg aggatoacti gaggicagga giicgagace agcigggeaa cacagiggaa 14040 coccatetet actagootgt autoccagot totoaggagg tggaggttge agtgagooga 14100 Estoatgoca cogosotoca gootgegoga cagastgasa otgogtotoa saassaassa 14160 satissasse catacagate sagatosatg gtgataceag sagatagges gtetggaage 14220 gosanceagg casestante tgtagenagt tetggmeetg etetgageet eaganteece gootgaceae trancitees estatttooc sestectrat rigaseaerct rriangiezo 14340 aggetticag tectecoage tittacette tectettest cotcaggors speciments 14400 gtaggtgtte ttoaccoset titegesace gesacteegs ctcagagetg tteagteett 14460 goocsaggic acaagciagi gagtaggaag gigggianaa cacggotgic agaogcogga 14520 gootgetgoo otttococca acaaccatac tgcagcagac agogtogggg gosgutggga 14580 14640 tgggcactgc cacagoccat gatggaaggt ggaggaagaa gtgctccccc agaggcaaca teggtoaggg casagaggtg goacgoacag agtocacatg ggoagtgoot ggoogtgagc 14700 asgocatgig gotggagess agtigticig agaggtgggg socotgotgt agagggtest 14760 tenaggecas gotgaganec canggatgas atetecaços geggaggect geocangota 14820 octggcagga mentgaccac agaogggate agggeocaga agactoagta ggaaccocat 14880 czeterego gaoagtgaca egatgtgoac tgeggeaggg geagacatga gtgtgagaag 14940 caegastcag cagggotagg gagocacoca cocacaaact ogcoogtact otgtaggoag 15000 gttgtcactt tttcttttct ttttttttt tttttgagat ggagtotete cogetgeaet 15060 caggotygag tgosgtgaog castotogge teactgosac etcegeetee tgggttosag 15120 15180 castictoot goctosgoot coctagiago igggactoso aggigogoso caotatgoos gootsattit tittitgtat cirtagitga gacagggtti caocatgitg gooaggotgg 15240 totignasto otgacotogi gatecaccig coteggecte coasagigot gggattacag 15300 gettgageca ccatgecoag ooggitgtes ettittettt ittetttega coggitgtes 15360 cttttottt ttotttoctt tottottteg actgggtgte actttttett tttotttoct 15420 tigitticit tetitecite effectiffe tilettetit eferciciti cettiffice 15480 ottottieto tiittetite tiecticest teetistite etiscotete tittittitt 15540 tttttttgac acagastott gotttatoec ocaggotgaa gtgoagtgte acaatocaac 15600 tonotronec giogeoptos tiggotones tentoctoct gostonect tecanetane 15660 tgagaocaca ggtgostgoc actacatoca gotatttatt agtagtasta stagtogtag 15720 togtagtaga gatgaggtot otcoctatgt tgcccagggt ggtottgaac tottgggoto augigatect occasoring octooceano igetaggact accaseaget igggocatea

[Drawing 19]

tecocagist tittitites tagagetggg gtototetet gitgoccagg otggittes 15900 actorigage tonagonate ttoccoctte ggeeteecan agtgetggga toncagnogt 15960 gogoczocto statogiceg gitticecti tomacatect agetecases tegitotasec 16020 tectetesco ctecctore tocactares toctotecca organizationtittt 16080 tgtttgttec cacattaggs ctttgtgete accateteet etgeocagas atgettgaet 16140 ctigateact acgicacate goosgotoot citoctocto atoogggiot osgotossat gioacciect egggaaggee teestgatee eteeagetag tastgioacc tgeacaccea 16260 gtcacttgct agcatatgas gctggttcat tttctttttt ttttttttt ttgagaceag 16320 gtotoactot gtogoccagg otggtgtgoa gtggtgoast ostogotgac tgcagocttg 16380 acctocoagy otcasgosay cototoacot tagoctotoo stagotggas ccacaggacy tetecoacce caccoagote atticitati tilistages ecaggetote actataties 16500 coaggotagt otosasoton taggotosas castostoto gootosacot occaçagtac 16560 tgggatosca ggtgtgsecc actacacocg gccaaggetg tetattttet gcgtaatact 16620 totgagasto tgosatgato castitatig ggitatitgi toggicigia soctoocaci 16680 greetstees otcostress scaressocs tetotatott ettoectoca stattaccas 16740 catottasac agoacotggt gostestage tgotosacec stacctetts satgastgat 16800 gonggggane gggangtgan agganocaen gangatggga connegtots stettgggan 16860 16920 gigogragas gittercinga cetegenger cottogoger ignoctotes oigcistatit tgaggtgttg tgagotacco agggggacag gtootaagca ggaggcagaa gcaggagccc 16980 agaggotgga agtagasatg castggagag aacagataca cacttgtoca ggggassatg 17040 ttesagaggg agcagaggag catgagtgag cogggggtoc tososcottg ogggaagggg sagttester gracerrass cargeronne generonnes ategeantes esticacaes 17160 ogtograpte agcatosace asgocotoco tocotocasa actascages gactgegaco 17220 ocgategteo cogggaette occaceggac agcatetete tetagggagg gggaagccac 17280 GOCGBAGGOC COCAGGOGGC GGGGCTGAGC CAGCGGCACA GCTGTGCACA GTGGCCCAAG 17340 CCAGGTGAGG AAGAACCGCT CAGTAATCAG CCACTTCCTC CTCGATCCTG CCTGCCAGGC 17400 CAATggcact goagggacae acoctggact astagtotoc agraeesges cagetgggo 17460 aggrotagec egagacegee cacageggee agetteectg eagoccessa gegggagtae 17520 agoaggooog octgetggte cagggoogce ttoccoccagg agaggaggag ccacctagag 17580 ctccscotex agetesaget gaoagagege organitatic estaggegas cagaccages 17640 agtacocoty gotgeggaag agoacoccat gegggaaacoc totocgtagg oggaaacoca 17700 aggreetsag gasastooct sacagoocag ttoctcagag saggaggtac occotgtgos 17760 MECCENCOCA OCCARGEGO tgagggacoo actoongeet eteaneetge cocargegona 17820

[Drawing 20]

EREESEGAEC	ctoaggtagg	gttaggccct	EESECREEET	taggoccago	caggaccata	17880
EEEAEEAECT	gttcaggggg	atgeggcctg	gaaaggcaga	gggaatcagg	tastatata.	17940
teggegttet	onggtoogca	agagactgac	accaaggeta	agtcacaggo	gcatttatta	18000
ttgtotggaa	ostosaggoc	tttootcooc	tggcagtggo	acaaggaagg	gocaactoto	18060
aggaggoggo	oacgotgoca	ocagoagoag	goocatgggg	tggcagggto	atgggcggca	18120
gaascagege	ottoagottg	octgacaggo	tggcgatete	aggatoctgg	gottogtagg	18180
aottgaccaa	gogagoasso	ttaaggacac	ctgosagagg	gagtggaagg	tosaccotto	18240
toctaaggac	cagcgtgoct	sagsoacctg	gagggggtag	tagtoteago	atgaactgct	18300
cocceactes	ctgggcagct	gtttgocasa	caggacgoat	gcaggoctot	gtocogtgtg	18360
ettteratra	tracatttes	totagactes	goaceacter	stee		18404

[Drawing 21]

	ATOCTOTOCO	STEGSTEGTT	TCACCBABAC	CTCAGTGGGC	TEGATOCAGA	GACCCTGCTC	60
	AA88GCCEAG	STGTCCACGG	TARCTTCCTG	GCTCGGCCCA	ETCECAAGAA	CCAGGGTGAC	120
•	TTCTCGCTCT	COSTCAGGGT	GGGGGATCAG	GTBACCCATA	TTCGGATCCA	GAACTCAGGG	180
	GATTTCTATG	ACCTETATOG	AGGGGAGAAG	TTTGCGACTC	TRACAGAGCT	GGTGGASTAC	240
	TACACTCAGC	AGCAGGGTGT	CCTGCAGGAC	CGCBACGGCA	CCATCATCCA	CCTCAAGTAC	300
	CCCCTGAACT	GCTCCGATCC	CACTAGTGAG	AGGTGGTACC	ATOGCCACAT	GTCTGGCGGG	360
	CABBCAGAGA	CGCTGCTGCA	BOCCAAGGGC	GAGCOCTOGA	COTTTCTTGT	GCGTGAGAGC	420
	CTCAGCCAGC	CTGGAGACTT	COTOCTTECT	GTECTCAGTG	ACCAGCCCAA	GECTEGCCCA	480
	OSCTOCCOSC	TCAGGGTCAC	CCACATCAAG	GTCATGTGCG	AGGGTGGACG	CTACACAGTG	540
	GETE GTTTGG	AGACCTTOBA	GAGCCTCAC6	GACCTGGTAG	AGCATTTCAA	GAAGACGGGG	600
	ATTOAGGAGG	CCTCAGGCGC	CTTTGTCTAC	CTGCGGCAGC	CETACTATEC	CACGAGGGTG	660
	AATQCGGCTG	ACATTGAGAA	COGAGTOTTO	GAACTGAACA	AGAAGCAGGA	GTCCGAGGAT	720
	ACAGOCAAGG	CTOSCITCIG	GGAGGAGTTT	GAGAGTTTGC	AGAAGCAGGA	GGTGAAGAAC	780
	TTGCACCAGC	GTCTGGAAGG	GCAGCGGCCA	GAGAACAAGG	GCAAGAACCG	CTACAAGAAC	840
	ATTCTCCCCT	TTEACCACAG	CCBAGTGATC	CTOCAGGGAC	OGG ACAGTAA	CATCCCCGGG	900
	TOCGACTACA	TCAATGCCAA	CTACATCAAG	AACCABCTGC	TAGGCCCTGA	TGAGAACGCT	960
	AAGAGCTAGA	TOGOCAGOCA	GGGCTGTCTG	GARGCCACEG	TCAATGACTT	CTGGCAGATO	1020
	GCGTGGCAGG	AGAACAGCCG	TETCATCETC	ATGACCACCC	GAGAGGTGGA	GAAAGGCCGG	1080
	AACAAAT6CG	TOCCATACTS	GCCCGAGGTG	GOCATOCAGO	GTECTTATEG	GCCCTACTCT	1140
	GTGACCAACT	GCGGGGAGCA	TGACACAACC	GAATACAAAC	TOCGTACCTT	ACAGGTCTCC	1200
	CCGCTGGACA	ATGGAGACCT	GATTOGGGAG	ATCTGGCATT	ACCAGTACCT	GASCTGSCCC	1260
	GACCATGGGG	TCCCCAGTGA	GCCT6GG6GT	GTCCTCAGCT	TCCTGGACGA	GATCAACCAG	1320
	COGCAGGAAA	GTCTGCCTCA	COCAGGGCCC	ATCATCUTOC	ACTECAGOGO	COCCATCOCC	1380
	CGCACAGGCA	CCATCATTGT	CATCGACATE	CTCATGGAGA	ACATCTCCAC	CAAGEGOOTG	1440
		TTGAGATGCA				•	1500
	ETGCAGA06 0	AGGCGCAGTA	CAAGTTCATO	TACGTGGCCA	TORCCCARTT	CATTGAAACC	15 6 0
	ACTAMBAAGA	AGCTGGAGGT	CCTECAGTO	CAGAAGGGCC	ABBAOTCOGA	GTACGGGAAC	1620
	ATCACCTATO	COCCAGCCAT	GAAGAATOCO	CATOCCAAGO	CCTCCCGCAC	CTCGTCCAAA	1680
		ATSTSTATE					1740
	AAGCAGCGET	CAGCAGACAA	GGAGAAGAGC	AAGGGTTCCC	TCAAGAGGAA	6TGA	1794

[Drawing 22]

N			C
SH2	SH2	PTPase Domain	C-terminal

[Drawing 23]

MVRWFHRDLSGLDAETILKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSG
DFYDLYGGEKFATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSG
GQAETILQAKGEPWTFLVRESLSQPODFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRY
TVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYYATRVNAADIENRVLELNKKQESE
DTAKAGFWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNI
PGSDYINANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVE
KGRNKCVPYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQ
YLSWPDHGVPSEPGGVLSFLDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENIST
KGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFIYVAIAQFIETTKKKLEVLQSQKGQESEY
GNITYPPAMKNAHAKASRTSSKHKEDVYENLHTKNKREEKVKKQRSADKEKSKGSLKRK

[Drawing 24]

```
actocctett geaggtgtee ttaagtttge tactggte aagteets aageocagga
tootgagat caggoagot gaagg ctg tttotgo coatgaccot
gocaccocat gggootgotg otggtggoag ogtggomoc tootgagagt tggcootooc
                                                                 360
ttgtgccaot gocaggggag gasaggcott gatgttccag acastestaa atgacctgt
                                                                 420
gactiegoet tggtgtoagt otottgggg cotgaceacc occatetete ettocetgat
                                                                 480
tocototgoo tttocaggoc ocateccoot gascagetco tecetatggt cotggetggg
cotssocots coccargeo tascoctaco traggetect eccettecce Eggeoaget
                                                                 600
tgagaggotg gagtgggtoc otcagarcoc tgagtgggtg ggcotgoaca gggggtacot
                                                                 660
cottototga ggaactgggo tgttagggat tttccttagg ccctttggtt tc
                                                                 720
gagaggitte ecceatiggt tgetetteet cagecagggt tactteetgg tetgiteece
                                                                 780
tacccaatac competents tgtoagettg agetocaggt ggagetecag gtggetoete
                                                                 840
ctetecing ggsage go cetggaceag caggacect tgotgtacte conttiggg
                                                                 900
gotgonggge agotgood c tgtggg gt ct gggcong coc cccca cotgtocttt
tectggagae tattagteca gggtttgtee etgeagtgee ATTGGCCTGG CAGGCAGGAT
                                                                 1020
MAGRAGGAA GTGGCTGATT ACTGAGCGGT TCTTCCTCAC CTGGCTTGGG CCACTGTGCA
                                                                 1080
CAGCTGTGCC GCTGGCTCAG CCCCGCCCCC TGCGGCCCTC
                                                                 1140
TACAGAGAGA TOCTOTCC TOGgtaagte coggcacca togggteec agtetootgt
                                                                 1200
tagittigga gggagggagg gottigitga igotcacto
                                                                 1260
atotgo ctgcoctg cctgttto gtccctatga acttcccctt coccaaggt
                                                                 1320
gtgaggacco of getoact catgetoote tgooccotet ttaacatttt cocotggaca
                                                                 1380
agtgtgtate tgttetetee attgcattte tactteeage etetgggete etgettetge
                                                                 1440
ctoctgetta ggacetgtee occtgggtag etcacasoac etcasacata geagteagag
                                                                 1500
gocacco asggoodtoc cantocogo casetteto caettecos sestesgeet
                                                                 1560
ttggtcccat cttotttgtt toctttoact tocctttocc ctgcatcatt cattoescag
gtalitette agoatotett etgosocogg tgotgittas gatgotggta etectggagt
                                                                 1680
gescasgecs georgetot otgototos gegottace thoosgtggg aggittecage
                                                                 1740
casacasat ascocastas attggatost tgcagattot cagaagtatt aaccasaa
                                                                 1800
tagacagoot tego megts tagtsettes cacctetest occasioning teggasgots
                                                                 1860
agginagage attectigas occaggagit teagaccase etescoasta tasteagace
                                                                 1920
otgtototac assassatasg sasttagotg ggtgtggtgg cacamitcct gtggttccag
                                                                 1980
ctatggagag gotaaggtga gaggottgot tgagootggg aggtcaaggo tgcagtcagc
                                                                 2040
gatgattgca ocactgoaca ocagoctggg acagagtg agaccttgto toassassas
assassasas gassatgasc cagottosta tgotagonag tgactgggtg tgoaggtgac
```

[Drawing 25]

[Drawing 26]

[Drawing 27]

ttatttagtt gigittagig tagattagat gitattattt aasttataog ggatagaggi 60 tigtatgogt ittigtitiggt anstagtigt timgttagtg ggggagtagt timigtitiag 120 attattattt tilttaggig tittaggigc gitggittit aggagaaggg tigatittit 180 attitutit gtaggigitt timagittgi togtitggit magittingg angittnega 240 ttttgagate gitagittgi taggisagit ganggogitg titttgiegi ttatgatiti 300 gttattttat gegittigtig tiggiggiag ogtggiogti tittigagegt iggittitti ttgtgttatt gitaggggag genaggittt getgitting stastesies etgegittgt 420 getttagttt tggtgttagt tttttgcgga tttgataatt tttatttttt tttttttgat 480 ttitttigit ittitaggit ttatttitt gamtagittt ttittatggt ittggttggg 540 titaatitig tittagggit taetitteti igaggititi tititititi oggggiaggi 600 tgagagetts gagtesettt titasoettt teggtesets settletata sessetatit 660 ttttttttga ggsattgggt tgttagggat tttttttagg ttttttggtt ttogtttaog 720 gagaggitti tittatiggi tgttittitti tagtiagggi tattititigg titgttitti 780 tattiantet ttogtogttt tettagttte agtittaggt ggagttitag gtggtttttt 840 tittittoggg ggeaggoggt tittggattag teggogggtt tgitigtatti tegittitggg 900 gtigtaggge agtiggtogt tgtgggoggt ttogggttag titogtitte titgtititt 980 ttttggagat tattagttta gggtttgttt ttgtagtgtt ATTGGTTTGG TAGGTAGGAT 1020 CBAGGAGGAA GTGCTTGATY ATTGAGCGGT TTTTTTTTAT FTGGTTTGGG TTATTGTGTA 1080 TASTISTETC SITUATITIAS TITICATITIT TOCOGTITIT COTOSTOSTI TITITITITI 1140 TATAGAGAGA TGTTGTTTCG TGGgtaagtt toggggtatta toggggtttt agtttttgt tagittigga gagaggagg gittigitga igittatito gaogigigig aacgigagig 1260 contitutes tigitities titigitities gittitiates attitititi tiegiasegi 1320 gtgaggattt toggtttatt tetgittitt tgtittitt tteatettit tiltiggets 1380 agtgigiati igittitti attgiatti tattitiagi ittigggitt iigittiigi 1440 tttttgitta ggattigitt tittgggtag titataatat titaasista giaritagag 1500 gttattogog seggittitt tacgittagt taattittito gtatilittia ateitagatt 1560 1620 gtacgigtig agistitatt aigistiagg igiigities geigitggie sistiggegi 1680 gastasgata gatatggttt ttgtttttac ggagtttata ttttagtggg aggttataga 1740 togastasat sattiaatsa attggattat tgtagattit tagaagtatt aogtagasas 1800 tagatagitt tagtogagis tagiggitta tattigigat titagiatig igggaggitg 1860 aggogagagg attettigag tittaggagatt igagattagt tigattagta tagtgagatt 1920 tigititiat assessins assitistis sgigtggigg tatacettit siggititis 1980

[Drawing 28]

ttategagag gttaaggtga gaggtttatt tgagtttggg aggttaaggt tgtagttagc 2040 gatgatigta ttattgtata ttagtitggg cgatagagtg agattttgtt ttaasaasaa 2100 sasaa gaasatgaat tagttttata tettagtaag teatteete tetagetgat 2160 attettegtt ggagggatta gggaggtttt ttogaggagg tgatettiga gttgagatto 2220 seetsesses seessessest testiatets sostestest teacesties statitities 2280 2340 gtagagaga tggtgagtat aaegtittaa tgtgggaata aatasaassa ggatagtgtg ttogtggtag aggattttag tggagoggag gtagggttat agtaggttag attatgttgg 2400 agitagrate tigasagies assittence agatempete ecetacetti etentittae 2460 tatttiggga ggtcgaaggg ggaagatigt tigagtitag gagtttaaaa tiagittggg 2520 testetagag agatittett titettesse sessetatig ggtetgatgg titesgistg 2580 2640 tggtagtttt agtagttigg gaggttgagg tgggaggatt atttgagttt aagagtttaa gattattitig ggtastatag ggagagatti tattittatt acgattacga tiettattat 2700 tattastass tagtterate tartertate tatttetest titagttatt temasette 2760 aggragage attettigag ttaaggaget ogacgitgta gigagitgga tigigatett 2820 rtattitest tigggigeta sagisegati tigigitesa sassassasa aacagagaga 2880 Leeklassin elkusiilse ilrainula sespialese leeklassen sulkasulai 2940 cgegetagen genegestag genggangga negnungun ngennggan genesaguen 3000 3060 sagtgatatt tagtogasag asgasaggas agasasagas asagtgatas toggtogasa gasassagas asagtgatas toggttgggt atggtggttt aagtttgtas tittagtatt 3120 tigggaggto gaggiaggig gattacgagg tiaggagitt aagattagit iggitaatat 3180 ggtgaasttt tgttttaatt seagstetss asessesatt eggttggtet egtggtgcgt 3240 attigigagt titegitett egggaggite eggteggage attritiges itteggagge 3300 ggaggttgta gtgagtogeg attgogttat tgtattttag tttgagtgta gogggagaga 3360 tittatitta sasassassa sasasgassa gasasagtga tastitgitt atagagtati 3420 geographite terrirerie ettititagi titetigati titettitti atatitatet 3480 tigititigt titlegigtat attitigitat igicggitti alogaiggg tittlatiga 3540 gttttttegt ttttgattto gtttgtggtt attttttgt taggtagttt ggttaggttt 3600 tttttggigt agattitatt tittggittit tagtitggit tigaatgati tittatagte 3660 3720 aggittitet tiittegeet estittetti tegitetete gittetitac gettegetet igittatgig gatitigigo gigitattit tilgittige tilaigitgi tilliggegga 3780 gistittitt tittetitit tattaigggi igiggiegig titatiitat iigititicga 3840 ogtigtingt igtagtatgg tigtiggggg eengggisti eggittoggo gittgategi 3900 ogtgitttet ttattittt ettiettegt tigtgettit gggteettet tieetettit 3960

[Drawing 29]

tgagtittag tilltgittt taesattggg tgestastat tisttaegia gggtiggitt 4020 gaggattest agtatastgt assagttggt agtattgase tittgitett tettegtitt 4080 tistatingt attigggage tattetteng titatitett aggogggget titangetti 4140 agagtagitt tagaattitt tatagattat titgittigt tigogittit agattgitta 4200 ttittitgta ttattatiga tittgattig tatggttitt aattittitt tittitgagac 4260 ggagtittst titgitgitt aggitggagt goggiggtet gettioggit tettgteett 4320 titatittit gagaagitigg gattataggi tagtagagat ggggttitat igigitgiti 4380 agtiggitte geattitigm titteagtgm tittittett teggittitt emagigtigg 4440 gettataggt gteagttatt gogtttagtt gtattittet titttgaget agggttitat 4500 tttettattt aggtoggatt atgetgetat sattatgett tattetagit toggttattt 4580 taggtitaag ogattittit attitagtit titaagtatt tagggttata gaggtgtgtt 4620 attatattig gitaasttit gtattittig tagagataga gittiittag gittiittagg ttettittaa attigetett asgisattia tiestittet tittateene telleggeti 4740 ataggogtga gitattgogt tiggittiga tititattit tattititig tilitaagga 4900 satattittt tittitgast tatteggist tiettitigt eattittegi tillitteggi 4860 tigittitgi itaisagasa aiggggaasa igattittai attaisaggi igitigaggi 4920 ttesetence togistetet gameetentt tetanettit stattetett sagstanege 4980 aggiagitit ttettittt gitamaggat agtagmagti gintittig titgagitti 5040 gittittegt tigstatttt tiegaggast tittigttitt tittatgggit aggangaggi 5100 gittagitag tittitites gigtogagit aetittitit tettaagies sittaggitt 5160 tgganggagt gttttggggt tagggtgtag tgggcgtttg gtgttgagta tggatttgas 5220 atttogtgtg tggttagatt tatgttttat gogtggggat gtgtetogga ttaggtatgt gtgtaggtgg etetggetge tgaggtgtgt gtgttgtget tgtgtgtgta tttttgtttg 5340 ogtetgetee gteggtigte tetetegget teggeegtig tettigtest teggtetete 5400 gttatggatt gacgagttig titgttgast attigtting tgttaggtat cgtattggat 5460 tttgsatatt tcgagatgag ogagagogit agogggtgtt tcgcgttgta gitagtittg 5520 tetetettit tittettitt tegogitggg ogtgggggtt togoggattt toggggttit 5580 gregtegigt ttaagtigaa geggiegita aagtiggati atgiaatasa titattaggi 5640 agattittia gggtatingi gagagaagaa aattagaast aastagaata aanagnaaga 5700 assassagig generatitit titigggree anniatiget gitigetett titineseeig 5760 ataatstast tattetesse seattesett tettesetti assettiitti tittittitat 5B20 ttgaegtega atatgtatae tgtttatese tettsagtat etsetttggt tttattttt 5880 tttittttt gaastagagt tttattgigt tgttaggtig gegigtagig gigtgatttt

[Drawing 30]

```
ggttcgttgt amttitggtt tittgggttt amgtgatttt titgttttam titttcgmgt
                                                                       6000
agtigggatt ataggogitt attattatgt ttagttaatt tittigtatt tittagtagag
aggregatitt attetettes traggatest titsattitt teattioste attostiact
                                                                       6120
ttiggttttt tasagigtig ggettatagg ogigagitat igogitoggi taatittacg
                                                                       6180
tttatatata tttatgtasa tagtatttag atogagatas agagttttt ttgtatttta
                                                                       6240
sasgittitt agaaattgit titagitegi atatitetti tiataaaggi asigiatgit
                                                                       6300
tettatetes tatttesses getatetega geatteegte tittitites tittetitt
                                                                       6360
tagitattia gittititti tiaggggaag tietiasisi gigittitta igiattitti
                                                                       6420
gitgagitgi tittittogi titggittgg oggigitgei gittgiatti ggmattatag
                                                                       6480
staggtagta tiatatatti tegisittag gettittagg attagttagt titgaggage
                                                                       6540
ttagitates teaegattit ettititagi tittaggaga taggittiti egiggagig
                                                                       6600
tiggggtagg gtagaggttt agggataaga attagaacgg atttatgtig atagggttgt
tingggitet gitgittett tittigitet egiggietgg eisentigie inigitggit
                                                                       6720
agaggagget attitititt titigtaagte tiggisaggit titlaattatt agtititigt
                                                                       6780
tittetggta gittittigg alanggeggt tittestitt igittitiga agittigagg
                                                                       6840
gtiggigiat aggagitica agiatiggit tiggastogg attgtitggg titgastiti
                                                                       6900
ggtattgtag tigattiett geiggettie ggteetgtit taeettitti gegtittegg
                                                                       6960
ttttttgttt gtasastgat saagstagtt tttgttttat agggttgtgg tgegasetta
                                                                       7020
attagataag giatgigaac gitattatag tatagogito ggiattiagi aggattiati
                                                                       7080
cgatgatagt tgttatcgtt attattgtta ttagogtggg ttagggaggg ttgcgtamaa
                                                                       7140
gtagttegte gaggaggas asstgtogte ggatogttte gettogtate ogtgaagtat
                                                                       7200
tattigggit iggagigigi saggistata igigititis itigisigit igitatatat
                                                                       .7260
gtgtastgtt atgittitga gittitgatt gtagaogtgt gggaagtggg tttcgttttt
                                                                       7320
stittingig tistitigit tigtititit tittitgitg igititesse ogagasgist
                                                                       7380
aagtgagttt tittaagggg toggtogogt tittittigt titogititg toggitgitt
                                                                       7440
LARGETTAGTO GARTOSTAGT TITAGAATTG GGATTATOGG GGGTGGTGAG GCGGTTCGGT
                                                                       7500
ATTEGGAGIT GTATTTGAGG TITAGTTTTT GAGTTTTTTG TITGTTTAGA TTAGTTGTAT
                                                                       7560
TITTITATIT TITACETTIT TITTITTITC GGAAGTITTT AGGATGGTGA Getanggett
                                                                       7620
tettatttac getagatagg aggtaagggt gttiggigtt tacgggattt ttttttattg
                                                                       7680
ttttgtttgg gtcgtttagG TGGTTTTATC GAGATTTTAG TGGGTTGGAT GTAGAGATTT
                                                                       7740
TRITTAAGGG TOGAGGTGTT TACGGTAGTT TTTTGGTTCG GTTTAGTCGT AAGAATTAGG
                                                                       7800
GTGATTITIC GITTITCOTT AGgtaggtgg gtttttcgta atttcgggta ttttggttat
                                                                       7860
tittitgigt tettiaggit tigeattett tattitiggt tittogiggt agigtigett
                                                                       7920
```

[Drawing 31]

```
tttogtttgt ttttttgttf tteettttta tattttttat ttttgtttgt gtttatttat
gittetgigt gittitatti aggettiteg togettitig tittitigti titettitig
tetogettes tittetogit testetitte tempercoes GATTAGETGA TITATATTOS
                                                                     8100
GATTIAGAAT TTAGGGGATT TITATGATTT GTATGGAGGG GAGAAGTTTG CBATTTTGAT
                                                                     8160
AGAGITEGTE GAGTATTATA TITAGTAGTA GOGTETTITG TAGGATCGCG ACGGTATTAT
                                                                     8220
TATTIATITY AAGTATICST TGAATTOTTI COATTITATI AGTGAGAGET SARRETTIOS
                                                                     8280
tattttogtt atttttaagt agggatgagt oggtitttat tittgeategt tagggaggta
                                                                     B340
seggesting instrugent intitatiti thattitit thittitist attentions
                                                                     8400
                                                                     8460
gtttttanig ittittttt tigitgittt gegattiggt gtittagagt tiaatttatt
stititita ittaatitos aggaagitat aggaagitei itogittiai itogggaatit
                                                                     8520
                                                                     8580
ttgstcgttg taetttaggt tttettggag atagggaggt tattgttggt ggttagtatg
tttogittgt ttittettit agtatetgit aggatagtga ggagtigata ttggggtgaa
                                                                     8700
gatggggatg aatgittgit aagatattig atgittigit tiagtogitt ogtggggatg
                                                                     8760
ggtttgtttt gtggggttam staggttttc ggtttammta gagmttattg agagtacgat
                                                                     8820
steamstett tattisteta austettita ostistitos estatasest astatittas
                                                                     8880
gtattittit titigiggitt titiogattit titigiggit titiaaaggi aigggitggg
                                                                     8940
ggttgggggt tttgaatgtt ttftatgate ttatggtttt ttttagtagt ogtetttes
                                                                     9000
tgttegettt ttttegegte aegggtegog gastemogtt agggggtttt tetatgtett
ttigggttas gtogettigt ttitgtogtg gattittgta titatggato ggitattgas
                                                                     9120
                                                                     9180
stgatoggga attitgitt tgitagitig tagittitit gagattoggg ttittasett
gtattaatat tittggitaa ggtattgatt gasatttaga gitggattog gitaoggigt
settitutes titatituse agettititi tititusatos stittititia agettititit
                                                                     9300
tttttttgtg agitttatat ggitggitto gtgtftgttt titgttittt ttttttttta
                                                                     9360
togtastett tagggggttt tiggtatoga gatittitas agtiteigti tittittiti
                                                                     9420
tittettiti agitaggagu ggaggacggg tigattagig tittggaggig gaagagaga
                                                                     9480
gtagggtttt aggaggtttt tgtagaggag gttgaggttt gggtttaagg agaagagaga
                                                                     9540
egagagagaa ggaagggagg gtagtgtogg ggogggaggt teagattagg gaagtogtat
                                                                     9600
tggeggtttt titgggtget togtittagg agttagtgtt attittgagt ttgggggtgt
stangaggit tittititte sgittigite tettititet titettigte cettititit
                                                                     9720
tttgcgagaa tttgtatttg tttttcggtg gttttgcgtt ttttgtggtt agtttggtat
                                                                     9780
tigtetggag attitutet titegggetti gagagaagag ettiagitti titticgita
                                                                     9840
tttggggttt tagttigtit ttaggoggtg ggttgaagta gtttagtggg gttaggaggt
```

[Drawing 32]

titurgenett titonetter agttattito gegtagenet gagetgggit gagetggatt 9960 ggtttttttt ttttttttt tatttttgcg gttggmamat ttgttogttt ttttttogtt 10020 tttgggttga ggasatttta tasttttatt ttttattttt tttttsgasg gagttttgtg 10080 ttittittet teorigetti titergeget teggettitet gegetteteg ttittitte 10140 ggsanggggt gtgtttoggg gasagggttt agttttgtt titgttttga tagtttttt 10200 assiticgitt gastitiggg tittittita gigatattat tiagggiatt tiagaattit 10260 ttatattatt ttttttttag tgggsttgtt tttttogttt ttttggogga gogiattita 10320 ttogtttttt ttgtgatttg agtttgtgtg tttatttttt attattttt gtgatgtgtt 10380 ttoggtttgo gttttttttt gtttttggtt tttgttgggg tategtttta ttttttaogg 10440 agatitatit tiagittitt tittitisaat attitgaata tigitagitt tittgittit 10500 tagasstres titteretto saesitoset tagastitta sagattagas tagittagat 10560 tigggaggio gaggitgiag agagitgias togggitati giattitagi tigggiasia 10620 gagtittega agtitettit agagtiagti eagggtitta ggitagtgag tastagtita 10680 gogttagttt tittatitat assatggggg taatattata titagtiitt agtatgiitg 10740 tgagegattt eastgagetg gtggatttgg eagtetgtag ogtagtgttt ggtatatagt 10800 aggigtitga iittoggiit tiittigiga aigililitgi itagogiiti tiittigiggi 10880 ttgggtttta tiltitiga ogitgittit tilagereet ATFATGETTA TATGTTTGGC 10920 GGGTAGGTAG AGACGTTGTT GTAGGTTAAG GGCGAGTTTT GGACGTTTTT TETGCGTGAG 10980 AGITITAGIT ASITTOGASA TITCGTGTTI TITGTGTTTA GTGATTAGIT TAAGGTTGGT 11040 TTAGGTTTTT CETTTAGEGT TATTTATATT AAGGTTATET GCSAGgtaag gtagttaggc 11100 11160 tigitigggt tigeatites ggtiggggat tiagggaggs sgattiaggt titetesate. 11220 gtttaattig gittittita gestegacet tatatastes steetitesa sattitceat 11280 AGTITIACGG ATTTGGTGGA STATTITAAS AAGACGGGGA TYGAGGAGGT TTTAGGCSTT 11340 ITTETITATI TECCSTAGET teggegters titagitett tittigitt tittegette 11400 ttttttagat gigagiitit gggattitig agiigiigat tittogiiti tittiatiti 11460 ARTICGIATIA TOTTACBAGG OTGAATGCOO TIGATATIGA GAATCGAGTG TIGGAATTGA 11520 ATAAGAAGTA OGABTTOGAG GATATAGTTA ABSTTGGTTT TTGGGAGGAG TTTGAGGTgt 11580 steategers togetagget tegggtagtt gamategtes tagggettte gestittege 11640 oggstatttt tittittitg titatittig titlitgatti sittisogig agittittog 11700 atgratettt tillteggag tigatetta tittittatt tatatittag AGTITGTAGA 11760 AGTAGGAGGT GAAGAATTTG TATTAGCGTT TGGAAGGGTA GCGGTTAGAG AATAAGGGTA 11820 AGAATCOTTA TAAGAATATT TTTTTTTgtg agtattlagg ttgttttatt tatttaggat 11880

[Drawing 33]

```
atogitting tittagingt tittititat tittataggit titattitit sogitaggag
                                                                    11940
12000
ttegesgigt tittitette ttegtaggte ggttgttttt tgttttteet ttttttgtge
attititist tittitists tagaigatit titatitits tigtitetes titticetae
                                                                    12120
gttttetggt tittgagatt agantggttt gitagtttag gagggtttga tttaggtgtg
                                                                    12180
gtgagttttt ggttaattta gattatttog ttttttttto gtttattttt agTTGATTAT
                                                                    12240
AGTOGAGTGA TITTGTAGGG ACGGGATAGT AATATITTOG GGTTCGATTA TATTAATGIT
                                                                    12300
AATTATATTA AGettagtag tgtgggttao gtgggaggag eggttgggtt ttgggaettt
                                                                    12360
titgtitggi ggggggatti tagattinga gatagtiggg tanngtogna gitggttitt
                                                                    12420
tgtetgggtg agggtggtag tggtttaggg tttgtgttgg gttaaggggt ttattgtttt
                                                                    12480
ggggtgogtt titttaggtt tgcgtttegA ATTAGTTGTT AGGTTTTGAT GAGAACGTTA
                                                                    12540
AGATITATAT CETTASITAG GGITGITTGG AGGITACOGT TAATGATITT TOGTAGATES
                                                                    12600
CGTGGTAGGA GAATAGTCGT GTTATCGTTA TGATTATTOG AGAGGTGGAG AAAGGTCGGG
tagggogitt tittittito giatiogitt togigitigi ggitatgita timagicese
                                                                    12720
gagtagttag atgitagggt agaaagggat titaggggtg agggttoggt tittgttege
                                                                    12780
sestigaggg tingigates agtitogett etetescgig attitisget ittigistgt
                                                                    12840
attitigggt tillligagt titlegatite ggtttteggt tgtttttttt tittitettt
                                                                    12900
tigititett igitigiett inggittitt tigittittt tettitetes ettititiss
                                                                    12960
agittgitti tiattitgia ggittittit atatagiati tittgigiig tiattgaagi
                                                                    13020
gettttattc gtgatatass ttgggttasg ttllttitt tttgasettt tttttatggt
ttttggttat ttttgggata aagtogtatt ttaeggttig gtattteagg tttggtggtt
                                                                    13140
tittitigat togtatgitt tittigaagg titatogitt tiagtagitt tagtittitt
                                                                    13200
aggittitiag tillittitig talaagiita tillitigita ggaaatgatt tillitatat
                                                                    13260
totttttgtt tggtegatgt ttogtttttg magnitudgt cggmgcgttg ttttttttgt
                                                                    13320
seattlesgt titgittitt tiaggattia gaggragaat tacgittitt tiagitacgi
                                                                    13380
ttittagege ggtgtttttt teggttattt gtttttgtga gtttttegag gtataggggt
                                                                    13440
stagattegg tettatttet ettigtgang tigtetegtt tetategtit oggegataat
gtitgtitte gtaacettig tigaetgata eacgestgta toggtgeagt gettegttag
                                                                    13560
gittistist itgitggigg tigattigag eogagegitt eggittitig tittittgit
                                                                    13620
agittattog ittatttaat aastgitteg gioggigita gginiitaga atatagagta
ggattiggga tgagttatag igittigitt igigititat tittaticga ittitittit
                                                                    13740
TEACATAAA TECSTTITAT ATTGGTTCGA GETGGGTATG TAGCGTGTTT ATGGGTTTTA
                                                                    13800
TITTGTGATT AATTGCGGGG AGTATGATAT AATCGAATAT AAATTTCGTA TITTATAGGT
                                                                    13860
```

[Drawing 34]

TITTTCGTTG GATAATgtga gtggttttta cgttttgttt tatttoggga gtttttttt 13920 sgettigtit tittittigg togggtagge tragetgest gagstettic gagagaguag 13980 ggggtattga tittatgitt toggtttagG GAGATITGAT TCGGGAGATI IGGTATTATI 14040 AGTATTIGAG ITGGTTCGAT FATGGGGTTT TTAGTGAGTT TGGGGGTGTT TTTAGTTTTT 14100 TOGATTAGAT TAATTAGCGG TAGGAAAGIT TGTTTTACGT AGGGTTTATT ATOGTGTATT 14160 GTAGgtgagg atgatesttt tgatggtagt agtgatagtt gagaagtaaa tettgttaag 14220 tgttetgagt tgttataagt astataaacg ttagttogta tattgagtgt ttttogtita 14280 tittoggitt tittigggit tittitatggi tittagaatti teggitggato giggitggaa 14340 ttegttttat tttrettttt tetttetege tettttttt eggettttt toggetgtet 14400 tatticetti sattigite asistagegg aggagitogg gettiagitg tiggitaggi 14460 ttaagttagt tagggtaagg togggtaggt attitatagte ggittgtgit toggtigitt cettttttt ceaestttte tittettest tittttttt aggastatit atsagstate 14580 tgittittat tittititt tittitatogg tagioglagg gittoggitt tittitigett 14640 tigittitit tittagitti tittaggiagi giritattit ggittitagg gitgigigg 14700 getgggtget gittittigg ggitgtetet aettititig titettiett ogtetgitig 14760 testtaggag attittegta aggigtagag gigggggitg taaggaggag taggggtitt 14820 14880 staggtgagt ttattgagtt ggtttggttt gggtggatga gaggtagtgg gtgtagggtt ttttcgttta ttagtigigi ggittigget esattetita atttititae ttittagtit 14940 ttttattigt aasattagga tittagggit giogigagaa titaatgaga tittatogit 15000 giggitgges titiogitegt tittassessi igggogitgi teliagitta giestitata 15060 tteggtagag aataggggaa tgggaatttg tritgtttog gittittit attittttog 15120 tagattttag atttaggacg attttagtt ttttttttt tttttagtag tigittatt. 15180 terestages taagtogett gaatttagag gtgttttoga teggttetto geggaagogg 15240 ttttgttttg tgttttttte gggataggtt tattttogag agitatttt ttgtttattt 15300 gitatetata tetitatata tittitigasa gittitatggi tittatitag sogitatagg 15360 saggaagter stategerege ttattittes tasttigest tigesatter stagogoset 15420 ttagggtatt agtitigtigg gittagtiga gegingettt gegettittt igaggittigt 154R0 tigittiageg itgeganagg agaganatit titetigiat igittitiig agittiitga 15540 tittgigttt togtattitg tigtittagg gitattitit tittgaogit agggittgaa 15600 RESEASORERS ESTESSETTS TETTESSES CETTITISTS TITLITIES ESTESSES 15660 gagggittag ggiattiggg agtoggiagg atagiggigg gaittiggggg tittiaggilt 15720 ttoggggtgg gegtagttat ttattaggag tgaggagtog gogogaggag tggaggagg 15840 saggatggig gtagtteggg agttagogtt agtatogtag agttogaggt ggagogtgit

[Drawing 35]

tetetegast temptement titettette titettoggt gettitgest statititit 15900 ttattettag agatttaggt tetttttata gigitttagag tiggagttag gogttaggta 15960 ttittittit ogggggggt tigstiggti tilgstggts tittogitti ttittagCGT 16020 COGTATCOST COTATAGOTA TTATTATTOT TATOSATATO TITATOGAGA ATATTITTAT 16080 TAAGGetgag gggtattigg gegitteggg giggegggig agtagittit oggigttogt 16140 ttetgtttgg atttgaggit tgattgtttt ttatttegGT TTGGATTGTG ATATTGATAT 16200 TTAGAARATT ATTTAGATGG TGCGGGCGTA GCGTTCGGGT ATGGTGTAGA CGGAGGCGTA 16260 CTATAAGTTT ATTTACGTGG TTATCGTTTA GTTTATTGAA ATTATTAAGA AGAAGTTGGA 16320 GGTTTTGTAG gtgogtgtag agtagggttt gggggggggg ggggttgtag tgtaggatgg 16380 stettattte ettitettes gattettett titttattet tittitetti ategTCSTAG 16440 AAGGGTTAGG AGTCGGAGTA CGGGAATATT ATTTATTTT TAGTTATGAA GAATGTTTAT 16500 GITAAGGITT TTCGTATTTC GTTTAAgtga gtggttttga ttgttattgt toggtattta 16560 tittitigit tigittagit ogattittet tittiggaga ggstaagigt igtagitggg 16620 gggattiggt itteegille ggittiggitt timitititt igittataag tattititga 16680 stattistac statesettt tigitassia timetascet attostatat sasatetast 16740 ttttgitttt taggagttig gagtttagig tagggatogi ggitgogita tttgigagac 16800 ggggtggtte geggggttg tiegtgtogg gttttlitgt gitgittitt gettigtett 16860 sattettet atttettitt tigtattogg tigtagATAT AAGGAGGATG TGTATGAGAA 16920 TITRTATATI AAGAATAAGA GOGAGGAGAA AGTGAAGAAG TAGOGGTTAG TAGATAAGGA 16980 GAAGAGTAAG GETTITTTTA AGAGGAAGTG ABCGGTGTTG TTTTTAGGTG GTTAT@gtmt 17040 agittititig tiligggigtt tittitigitt igittigigt tittiggitti atigittitit 17100 ttegeteget exectegics testitiett tigtetitti tegitetitt agattittit 17160 gttttetttt teggittteg ttattttitt attitttat tittttittt tegtegTTT 17220 AGITTITGATT TTETBGAAGT ATTTCGCGAT GGATAGATTT ATAATTTGAA TTTAGGAGTG 17280 TITTATETTE TETAATTIA AATGGTTGTA TITTETTTAT TITTTTEGA TITTGTATAT 17340 AGTTTAGTTA GGTTTTAGGT AGGGTTAATT TITTTTTTT TGTAAATAAA GTTTTGGGAT 17400 TATTetetet cetttttese ttttttett ettlagtes teggogetta gaggetages 17460 taggatgggt aetigtgigt gittitogige gigittegeg igasagitte gittitogit 17520 spaceseert pretogreet ticetticet acetergage steatogies etsaagtitt 17580 ttagtttttt ttittassat gragggogat tataatagag tegttetgan aagtatogag 17640 atgaoggitg acgataagac gegtatagig attitatiata cettigitat gigittaggi 17700 stissassat tatataogit agiltagilt aggletlitt gitattitta tittatogig 17760 goggasatty assestages esetteagte attigettet tigtttaage tistaggett

[Drawing 36]

atggestagt	gaggttggga	ttcgaattta	ggttgtttga	ttttegagtt	tatatttttt	17880
attttggagt	tgtagttggg	gttattttta	SESSESSITE	gattetattt	ttttgetgtt	17940
gagttttaga	tttgeattas	gaagagtagt	tee tegtogg	aagogtaget	ttgaggttag	18000
ttoggttgcg	tttttttgg	ceggaatage	gatagetttt	ttagagtatt	ogggtaogtt	18060
tagttttttt	ttttatttag	stestistis	tttttatttt	tttgggtaga	gtttgeageg	18120
ttggttgeog	tgeegegtgt	tttgtttttt	gttttttttt	tttttttta	tettagenet	18180
egegtttttt	ttttetttge	autattggtg	ttttggggeg	teasgtoggt	gggagttatt	18240
ttttaggaag	tgttggogtt	tatttttgga	aaggttgaga	tegtateggt	tttacagttt	18300
agaggttggg	ogtgtattat	ttagtanatt	tttatagegt	tttcggogtt	ateggtattt	18360
ataptitite	98111111 PP	astttargar	etteettase	grega		18404

[Drawing 37]

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tttttttgat tagtttttta agttttagga ggtoggggga ttgtgaatgt ttgtgaagtt
gggggtttig tenggattig tigegteetg teogtitegt tittgggtti tgggettigt
gttgitttag tittittagg agtgggogtt agtattitit gagggatgat tittatoggt
                                                                      180
tttetttttt aggetettag tgitttaest sangasgass ttttettttt getetggggg
                                                                      240
seggesgagg ggmtessess tesegtatti titteogiteg tingtittit ageittigit
                                                                      300
tengaggata agggtagtag oggtttegat gaggggagg gitgggogtg ttogggtgtt
ttgaggagtt tettittett ttcgttagag gegacetagt capatiggtt ttaggtttec
                                                                      420
gtiticggit gitaetteit titittagit tagattigga atttagisti aggggagigt
                                                                      480
gattegggit tittigaggg iggittiagt igteatitta gggtaaggag igigggitti
ggggttegat agtitgggit ogsattiteg tittatigtt tistegtitt gtgattitgg
                                                                      600
gteagtgatt aagttattta gtttttttgt tttttagttt togttaoggt aanatgagan
                                                                      660
tgatagaagt gittagattg auttaucgtg tgtagtitti tegtgiitgg giatatggta
                                                                      720
agogtgigat gagitattgi gitogittia togitagiog tiatitoggi gittittata
                                                                      780
attettitgt tatgategtt tittettita anganggage tiggggagit tietttacgg
                                                                      840
ttattittt acgigogagg oggagtitog atitacgito gittgacgga aggoggagti
                                                                      900
960
tttatttatt gggtaagtea agggtttaga ggogatetat AGIGATTITA GGGTTTTATT
                                                                     1020
TATAAGAGGA GAAGGGTTGG TITTETITGG GGTTAGTTG GGTTATATAT AGGGTTAGGG
                                                                     1080
AGABGTGGGG GGGATGTAGT TATTTAAATT ATAAAAGAAT GGGGTATTTT TAGGTTTAGG
                                                                     1140
TTGTGAGTTT GTTTATCGCG AAATGTTTTT ATAGGGTTAG GGTTGAGGTT gttsagagag
surastgags castgagags stagttegas tittggaggts gastaagags stittgggsta
                                                                     1260
gttgggaagt atagaatgag gttgoggtta tittattiat ttagggaagg tagtggagtt
                                                                     1320
eaggatatag ggtagggtag ggaggatatt taggtagang agttgtatTA TGGTTATTTG
                                                                     1380
AGENTAGIAT CETTIATITE TITTITEAGEG ANTITITETT TITTITTE TITGITEATC
GITGITITIT TATTITITIT TITTETTTET TITTAGTGTE TAGGTTTTTA TATATATITT
                                                                     1500
TITIGIGItt gtagtogget gtaggggggt aagtstaggt agttggtgta ggttaggaga
                                                                     1560
tagtataggg ggattoggta tiggtagitt tittiggite titogitita taggigaogt
                                                                     1620
egiteoggit titgiatteg attitaagit titagaggat ageggitate tittatetec
                                                                     1680
gagtgogttg tiggtatita gtagaggttt atacgtgtgg gtatttagga satgittatg
                                                                     1740
sategoaggg gigageatte egittigeatt igeogytegg tittitiegt igtestetti
                                                                     1800
gttttttttta gassgigage atogggitige giaggatane ggggiggetg togggiagig
                                                                     1860
gtagttaggg ttatttatTT GGACGAGGTG CGGGAGGTTT TGGTATGGGT ATTTTTTATG
STEGGGGAT AGSTGATGTT TTOSTATITC GATTITIEST STITTTEGGA ttgtgggtag
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[Drawing 38]

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agggatagig ggaaggiggi ggittiagia gggitaggig giattiatti igiatigiag
ttttttttt ttttaggttt tgttttgtac gtatTTGTAG GATTTTTAGT TTTTTTTAG
TESTITIAAT GAATTESECS ATSSTTACET ASATSAATIT STATTSCOTT TTCSTTTSTA
                                                                                                                                    2160
TTATETTCGA GOGTTGOGTT CETATTATTT GGATGGTTTT TTGGATGTTA ATGTTATAGT
                                                                                                                                    2220
TTAGGTtigg giggggggta gitanattit aggittaget ataggcggat atcraggest
                                                                                                                                    2280
tgtttettit ttattittae atttttaggt gttttttatT TITGGTGGAG ATGTTTTTA
                                                                                                                                    2340
TGAGTATETC GATGATAATG ATGGTGTTTG TGCGGTCGAT GTCGGCGttg gggasagsog
                                                                                                                                    2400
eggstgitat tegaggitag tiaagtitit ticgggaagg geggtatita gogittagit
                                                                                                                                    2460
ttegttttag gististagg agiagittge gitttiagtg atgggaggga atgtgittag
settatores teartraine teregettie titagittie teteratace tittatitice
                                                                                                                                    2580
ggttttgogg tgttgacgtt ggtttittag ttgttattet tttttttttt tttatttttc
                                                                                                                                    2640
                                                                                                                                    2700
gogtogatti titattitta gigagiggit gittitatti oggangatti gggattitta
settitutte tigititigio ggittittagg tattitgagt tittitigge tattitigge
ggagttetgg agcettittt agtetggttt tettitittt tittitigag tilligagett
                                                                                                                                    2820
                                                                                                                                    2880
aggesagga tagtitigag stagtagggt gogggggtat agggttaggg gattlaggg
agtagtgtag taggaagtti ittittitit tiagtitigg giasetagat ittaggaga
                                                                                                                                    2940
ttttaggttt attittagtt gagtttagta agttgatgtt tigagtogog tigittaatt
                                                                                                                                    3000
ttesettiag etigiteese steattittt stettiett tittitigte acettieset
                                                                                                                                    3060
sneggitets gggittitse geogtstyte estytytyt iggoggstga ginggaggit
                                                                                                                                    3120
agtitioggs gatgagitty tittigagag agtataggat agagtogogt titoggatag
tttatogene statttting atttagtoga titettttet titaggatee stagttatte
                                                                                                                                    3240
REFERENCE SESSEPTION SERVICETORY SESTIMATE THE SESSEPTION OF THE PROPERTY OF T
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                                                                                                                                    3360
ggatoggggt saggtaggit titettitit tattititgt tiggtgtgag tistteastt
agteetegog titlegittit gagggitgeo ggastittag tistesogat agggittist
tgagtitita ogstastiti gagettitga tittatagat gaggaagtig agggtiagas
                                                                                                                                    3480
sagttaagte attigtites gattatetag tiggtaagog gaggggttit gtatttatig
                                                                                                                                    3540
tittttattt attiaggita ggitagtita gigggillat tigiggamit titigititit
                                                                                                                                    3600
tttgtagttt ttattittgt attitattag aggittittg attataaata tgogggtaga
                                                                                                                                    3660
tagategagg estimizatet agtittenag sagtatiatt tetititata tagtittege
                                                                                                                                    3720
getteggetg gggtatigit tggggeagtt gggagagagg gtagagttag gaagaagtog
                                                                                                                                    3780
 segtitiges ettategate gasassegage eggestegge egtatatett tietegatet
                                                                                                                                    3840
 ttttgggaga agasattaat agastgggat ttogagagas agoggagtag togggatata
                                                                                                                                     3900
getttettet gestetttet togettttet titgettest tigestites tigestites tigestaatte
                                                                                                                                    3960
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[Drawing 39]

ggtttogggt tttttttttg tatttggteg ggttgggoge getggtatet toggagaggg 4020 ttttgeggae getatttete ggtegegggt temestgegg ttggtttteg ttacgattte 4በደሰ tttagggttt tggagttatg aggggattta ggagaagtog ggggtgagog gagggtattt 4140 satgigogag timacgitta tattgittat metagittat ggiattingi egiattiati 4200 tittagtigt tattattatt attaggatta ttattittet TIGTAGTGTA GGATGATGGG 4260 TITTECETEA GETAGATITY TITETCETTG ETTGATITGE TITAGGAAGT TGAGGATATT 4320 TITAGETITA ITGGGGGATIT TATGGTCGGG TTAGTTTAGG TAITGGTAAT GITAGATTTT 4380 TCSAATTAGG TTTTTttasg togsggstat agggttagtg tttttttttt tttcggsata 4440 tittattiat ittattitat tegattagag aggagastas gittagggag ggattitegg 4500 setgaggtag ggogtggggg ttatttatAT TGYTTAGCGG GGAGATITGT AAGGTACGGA 4560 GTTTGTATTC GETTGTGTTA TGTTTTTCST ACTTGCTTAT AGASTAGGGT TTATAAGTAC 4620 STIGITATETT TATTICEGET TAGTATGGGA CHTATTIETT tigghoung angetomet 4680 ggggatgagg tetegagtag ggtattgtgg titattittag gilligitit atgittigag 4740 tgtttggtet oggttteset etttgttgge tggacggetg ggttggtege geggtegeng attigggitt tegittiage tiaattatte ateggiggig eggittiggit egitatitte 4860 toggtatett cettigitat tisatassog tigitagggt aggtatigit ticgaagitg 4920 tgtesettet ategittitet egeteteset entettiegt tigigittit gigitticger ADAN gagittatas agatasetsa togggggaga tatogogitg aggagogtgg tteggaaaga ogtastitti tittiaggit itggaggasa tangattigg attiotagag gaggtagogt 5100 ttoggttgtg titttassas ogsggtattt gttaggtage getegtetgg agegagttat 5160 tttttegtag sematgagtt tgtgtomaga saggtiggge atttgemaga gttggggttg 6220 tigggggcag tgagittia agagaagogt gogggttaga gggaagitat tagattiga 5280 atgiteggit tiagagtece attitattit asaggteatt aggagtiate gassagutit 5340 tagesegges ggmattigat tiegtitigig tieoggaigg gettettite atggingtat 5400 egagggtgtt gigteggggg egittgiegg gineggggie getttiegeg egattietgg 5460 5520 gatagiting estingent temesting assenting gentaletet escretting 5580 gggttaogtt gtgtagtoga gattitgtta ttagttitta gttittiaat aagegtogga 5640 tittiatiti igagattiti tittigititig giattigati gittitogat itaatggiat 5700 gattataagt sogggggogg stgcggggas gggggggogt tttatTC9GT TTTTTTAT 5760 TTTTCGGGTG GTTATGACGA TGATACGGTT GTTTTTTTGT TACGTTATTT GTTAGAAGTE 5820 ATTGATCGTG GTTTTTAGAT AATTITGGTT GGCGATGTAG GTTTTAGCGT TTTTATTAGG 5880 GITTAGTAGT TGGTTttggs ogtasgogtg gagagacgte ttttaagsta gtgagttitt 5940

[Drawing 40]

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tggtttagta taggttttga attattgtta tttltattta tgtasgasgt tagtttcggt
                                                                       6000
titettiagt tettitigga tittaggetti tittlattaga tagggaatti titaggettia
                                                                       6060
gttttttttt ttacgtggtt tatattgttg etTTTGATGT AGTTGGTATT GATGTAGTC8
GATTCGGGGA TETTATTGTT TCGTTTTTGT AGGATTATTC GGTTGTGGTT AAttgggggt
                                                                       6180
sescesses sessosesat settingett estingent tiettatatt tesettasat
                                                                       6240
ttttttgagt tastaggtta ttttggtttt agmagttatg aggtttgogg gagattgtgg
                                                                       6300
gtagtaggag tgagggatta ttigtatgga gagatgagg gagttiataa ggaggtiggg
                                                                       6360
agtagggggt eattigting tiggtggtgg ggoggtettt tigggggggg tagaggittt
                                                                       6420
tiggagaagg gggaggitti gigggggig iggggagatg gittittitg gogiggaggg
                                                                       6480
tggagettig tgagetgagg ggaggtagtt ggggtagggg oggtattitg ggtgaatggg
gtegtttggg tgtttatAGG GGAGAATGTT TTTGTAGCGG TTTTTGTTTT TGTTTTTTGG
                                                                       6600
TCGFTGTTTT TTTAGACGTT GGTGTAAGTT TTTTATTTTT TGFTTTTGTA AATTELgaga
                                                                       6660
tgtgggtggg gesatgagte ttagttttte aegagggtat ttatoggggg agtttaogtg
                                                                       6720
ggsteggtte ggagtagagg teggstaagge geggsaggtg ttogtttggg gitttaggto
                                                                       6780
gtigttatta ttitagtigt titagtitta toggittita tiatgiatii TAAAITIIII
                                                                       6840
TIAGAAGITA GITTIGGTIG TATTITICGGA TITTITGTTIT TIGTITAGIT TIAATATICS
                                                                       6900
GIFFITAATG TTAGTOGTAT ITATTITCHT GETATAGTAC EGETEGEGET EGGBAGAGOG
                                                                       6960
agaagttagt satttagaga titttagaagt timtatitgg gggatagtit aggagaagtg
                                                                       7020
gggggtagt tgggtttatt tttgatTTGT CGTAGGTAGA TAAAGGCGTT TGAGGTTTTT
                                                                       7080
TTAATTITCG TITTITGAA ATGTTTTATT AGGTTCGTGA GGTTGTCGAA GGTTTTTAAA
                                                                       7140
TTATTTATTG TGTASCGTTT ATTittggggg gagttamett aggttattta taggatttga
                                                                       7200
gttttttttt tigggttttt agtttigaat timagiting gingettitt titgttettt.
                                                                       7260
statigiggt tatagatege agtittagta gaggittitt catogitteg tigititat]
                                                                       7320
TOGTATATGA TITTGATGTG GGTGATTTTG AGCGGGGAST TTGGGTTAGT TTTGGGTTGG
                                                                       7380
TYATTGAGTA TAGAAARTAC BAAGTTTTTA GOTTGGTTGA GGTTTTTACG TATAAGAAAC
                                                                       7440
STITAGGGIT COTTITIOST TIGHAGIAST STITITETTI STICSTIAGA TATETEGITA
                                                                       7500
TGGTATTATt tagagaaggt agcgttaggg aaggtaagat ttaggttata ggggaaggog
                                                                       7560
ttgagtagag stattistag agaggggtog gasattaagt atttatigig igitaggist
                                                                       7620
tgogttatat gtitttaaat ttattattit atttaggtit tttetesate tgitgagagi
                                                                       7680
taggtatgat attattitta tittatagat gaggamattg acgttgagtt gitattiatt
                                                                       7740
gettiagget tittestiga tittaggets agtittiags gittigtigt tiaggitggs
                                                                       7800
gigiagigge geggitatag tittitigiag tittogatitt tiaggitiae gitatitiag
                                                                       7860
tittiagagt tittaetoggg tittogaatti agagtitett tittgasaggi agaaaggitg
                                                                       7920
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[Drawing 41]

gtaatattta sestattigg aggagagag ttaaggetge attitogiga aggatgegat 7980 tgtgttttag tegagetteg aggtasageg seacgteget ogegettete ttatagggeg 8040 8100 suggangong spangataat titattgggg mangagtggt gtagggggtt tiggggtgtt 8160 tiggatgatg tiatigmagg ggagtitagg gittamaggg attigmaggg gitstiggag 8220 tagaaagtag mattaagtit tittitogas gimtetitit tittiagsag saastistag 8280 tittataang titagitita taggamette ogigetggen annatataan attitititg 8340 gggagagagt gagaagtgag gtigtgaggt tillitagtt tagggaggagg gggagggag 8400 gtasettitt taatogtage gatgggggga aggagggag gattagtite tittaatita B460 ttitettitt gtioggaggt gattitagto gagagattit tagagittit taettitett 8520 gggttatttt agtttetogt tiggggatag gttaggattt taggtagogg ggagggggtt 8580 gegtitittt tittaggitt teggetgagg sagtitttet gtacetgite ggttgettet 8640 aggaegogta gagttatoga gagutagata tamattttog tagaggagga ggogtataga 8700 tanggtagag gatatagtag nattigggag anagagitti tistatatit tinggittag 8760 gggtgetett ggtittigge acgggttatt temmegggit ittagtgogg tittittiggt tttaatittt ogtitoggia tigittitit tittittit tittititt tittititga 8880 atttaggttt tagttttttt tgtaggggtt ttttggggtt ttgtttttt tttttattt 8940 taggtatteg ttagttogtt tittttttt gettegaget aaggagaaag agaggatatg 9000 ngittingag ggittoggig timemegtit titgagigit goggigggga agagganggg taggregtes stackeegtt agttatetes getttetage gegaggeeg gitttesage 9120 sggtogettt aggmaaggag gittittagg tgggtistag ggtigtatog igatogaatt 9180 tegittiang tittesting tgittigett assessettig sigtagitig geggticges. 9240 ttttagagag gttgtaagtt ggtaggagta aggitttoga ttattttaat aatogattta 9300 tgantgtagg getttagggt auggstaggt contitentt taggggtag tetgangett 9360 ttttagogtt atttogtigt tttttatttt aaggegattt ggtattgaga tgcggttgtt 9420 gasaggagtt eiggigttet gaggagtett tagagittit agittitegt tieigittit 9480 sseasattat aggaggagto ggggaggtta tagganggas atgittggag taltattitg 9540 tgttogggat agortgagat attituteta extraetati ttetatogia ttittaatea 9600 tttttgttig ggloggagat ttattigatt ttataggata gattlattit tacgggggg 9660 tigggateag gistisagig tittggiong tettiatitt tettitistt tingigitag 9720 tittitiatig tittaataig igitggggig aggggiegat ggagganggi aggitogati 9780 ttagaggaag aagagttitt tastastaga gitggitigi acgatatgit gettattagi 9840 agtggtttt tigittting tgggattigg gttgtagogg tlagggtttt cggagtaggg 9900

[Drawing 42]

ogeggtagit ittigtggtt ittioggggt teggtggsss gggtggtagg tiaggttitg 9960 agatettagg tittaggeta giaggeggga gegutettga gagtittagt igetetagge 10020 agggggggg atggagggta ggtagtgttg gttgttagtt titttgtitt titggttgtt 10080 tagggtggga gtoggtttat ttttgtttgg gestggoggg ggtgcggagt ttttatTTT 10140 TATTAGTOGG ATCHGAGTAG TITAGCGGGT ATTTGAGGTG GATGATGGTG TCGTCGCGGT 10200 TITGTAGGAT ATTITETTET TGAGTGTAGT ATTITATTAG TTTTGTTAGA GTCGTAAATT 10260 TITTITTE ATATASSITA TASAAATITI TIGASITITG GATICSAATA TOSSITATIT 10320 GATTITIAT Ittgtagggt attaggoggt gaggttagte ggtgtaggag tagaggtagg 10380 aggetagega toggttgagg tittgggtgg gggtetetet gggtateget gggtetesest 10440 10500 aggratuage agtgtggggg ttggggggtaa gggaatagat ggggagttag tattgttaog aggasttage astgagtggt ttagggittg gatggtates gagagtggtt assatgttog Eggttgoggg gggtttettt atTTGACGGA GAGCGAGAAG TTATTTTGGT TTTTGCGATT 10620 GGGTTGAGTT AGGAAGTTAT CGTGGATATT TCGGTTTTTG AGTAGGGTTT TTGTATTTAG 10690 TITATTGAGG TITCGGTGAA ATTATttggg oggtttaggt agggtagtga ggaggggttt 10740 ogtgegtatt aggtattitt gittittgit tatogigggi ggtaggitti tatTTTATTA 10800 TITTOGOGGT TITCOGAGAG GAAGGGGGCG TAGGGAATGA GGAGGTGTAG TTAETTIGGG 10860 TAGGTAGAGA GTTTAGGGAT TAAGTTTTAG ATGTAGTTTT TAGTGTCGGG TCGTTTTATT 10920 ATTITCGETG GTTTTAGTIT TGEGGTTGIT ATTITATTES tttggggtag toggtagggc 10980 ggggategga egeggogogg togattitt grangaatti attigtetti tiogitting 11040 agtetagtas gggaagagaa gtagagtagg gtggtattgg gggtggggao ggggtttett 11100 tittateogt tigtaettaa aggittagga gtatggtatt gtatetetgt gataetatet 11160 gtagtaegga tatatgtgtg.ttttgtatat tttaggttta gateetettt tecgtatgcg. .11220 sattingang gillingget attititit tittining tigititian giagittit 11280 tiggittace timeteetee treingonet gataetigit atogastgas tittetiggs 11340 igicgggogt igigitates iggogittet eigititgit igetiggitt ittetteteg 11400 ttttatgasa taggggitat ttttatiati ttatagataa ggaattigag gittagggag 11460 titaaggtat igitigagit tattagigag tiagitgiag igitaggatt tasatitaga 11520 tagttoggtt ttaaagttag tgttttgaat ttttatatat tagtttttag ggttttaaag 11580 antagagatt angagtittt tigittamag gygtigitat gggagtagga gattantagt teagettitg timatgitig teagagagae gggtgtttit titteatiae tetatgiegt 11700 tigittatgi tattgiggis gagggatgga tantatgati tigagtagii tigitaatsi 11760 gggttttttt taattittet tittaggttt tigittigit tiagtatitt tattaggggg 11820

tttattittt gggggttggg ggataaggit titattetgg ttgattittt taggettegt

[Drawing 43]

```
tgattttaga ggitttaaat attaaggist etgatgitgi ttattigisa tiitaastat
                                                                      11940
anatattaat atogitasat tasaacgagg asaagtagti tastagggga tatataagan
                                                                      12000
statetatig giggittitt tiegggaggs sentigests sittggagget egggittess
gagatettig gittitiete tettititig eatgitatat gatgggtetg tettattitit
                                                                      12120
atesasatas etatettaat teggastaat titteeessa tittiaget etagerasee
                                                                      12180
tttittgttt ttstttggat gttgtttgta tgggtgtgta taeacgtgea gttggtoggg
                                                                      12240
ogtagtggtt taogtitgta attitagtat titgggaggt taaggtigge ggattaogag
                                                                      12300
gttaggaget ogagattett tiggitaeta tegigaantt tittititet teasanteta
                                                                      12360
sassaattag tigggistigs taginggogt tigtastitt agitatiogg geggitgeag
                                                                      12420
taggagestt attigsatti aggaagtoga ggtigtagog agtiaagsti atattettgt
                                                                      12480
attitagitt ggicatatag taagattite tittamesen aannamasaa tegagitags
                                                                      12540
ttgtgtgttt astatttetg astattatgt atgttttgtt ttaggtages gengenesse
                                                                      12600
ttttaagtit aeteagtite attitittet gatauttata tiettatitt tagaaatatt
                                                                      12660
asstattast gittititt assassaggit tilliettit tittititt tittititt
attigittit ggittittit tittettggt gilligggga ettigittgg tgcgttatt
                                                                      12780
gtetggitte gittigette tittittegt tiggetette tittegegit togganetto
                                                                      12840
goggaattit taogittaac gitaaggagt aagsagagta tatgtaaagt tggitgtagc
                                                                      12900
gogggatatt ogtiggogti tiogtitatt toggagtgit tagagtitag tgogatgitt
                                                                      12960
ggtatigagt sagigtitas tasatsagtt ogttagtite tegttatata titggttata
                                                                      13020
agtstegttt titiggtitta tatatatagt tigttigite teogtaggta agggtgtata
                                                                      13080
tetagitate giziatatat ittiattatit sigittatit sistetatat tiggitoggi
                                                                      13140
gtatattitt acgtatggaa tataaatttg attatataog aegitttagg titatgtita
                                                                      13200
gtattamacg titattgtat titggittia againtitti titagggitt aantitelli
                                                                      13260
sgtgggaagg aattaattog gtatttagaa ganattaatt aggtattttt ttttgattta
                                                                      13320
tggsagaggt aggestitt ttgsagggtg timegitemg agstagastt tagstaggag
                                                                      13380
statasttit tettattitt tegtaggama atamammati attittitta tittestate
                                                                      13440
strigggit triaggitat tittetatet acceptitiat tiaggitita gatestitte
                                                                      13500
tgatgtagga attattttt ttatttttt atgggoggaa ataagogtag ggagettsag
                                                                      13560
sattateaga gtemetgitt gutestting magamesses tattittitg gamgiagges
                                                                      13620
getassagte gagatteegg tteggogteg tggttteogt ttgtastttt agtattttgt
                                                                      13680
gggggtgagg tiggtggatt atttgatett aagtttgaga gtagtttagg aastttggag
                                                                      13740
acattttgtt tittatammae stetsmeett tegttaggig tggtagtets tittigtagt
                                                                      13800
titaggtatt teggagattg agatgggagg atogtttgag titaggggtgg togaggttgt
```

[Drawing 44]

agtgagttat ggtigtgtta tigtaattog gttigggtga tagagtgaga ttitgttita 13920 assastaces stategitgg scategigst tietatitgt astitiegte titteggagg 13980 togaggtggg aggattettt gaggttagga gttogagatt agttgggtas tetagtggas 14040 ttttattitt ettagittyt aattitagit tittaggagg tygaggitgi agigagicga 14100 gattatgita togtattita gittgggisa tagagigasa titogittia anassassas 14160 entiageset totalegatt sagettaste etgatelese sagetagete etttersage 14220 gtenstengg tesentestt tgtegenegt tittggsettg tittgegttt tegestttto 14280 gttigateem tgegtttest estetillit sestetiget gigeseegtt ggiesgiggt 14340 agggitting igitgitegt tittetette tettetteet titteggite ettitette 14400 gteggtgtta titatiteat titagaaste gemetteege titagagete tiaagtaatt 14460 gittenggit ataagitagi gagtaggaag gigggiseen taoggitgit agacgicgga 14520 gttiggtett tttttttta ataattatat tetagtagat agogtoggag gtagatgaga 14580 tegetettet tetaettiat entgesaget generases etettittit apportants 14640 tegettagge tasagaggte gtacetatag agtitatate getagtett agtogtgagt 14700 sagttatete ettesagtsa settettite agageteres stittettet agagestat 14760 ttaaggitag gitgagasat taaggatgaa attigiatta ggggaggiti ggitaagita 14820 ttiggtagga aastgattat agaogggatt agagattaga agatttagta gasattitat 14880 oggiggggto gategigata agaigistat iggggiaggg giogataiga gigigagaag 14940 teagesttag taggettagg gagttettte titteteestt ogttagtett tigteagtag 15000 gtigttettt tittittitt tittittitt tittigment genetittit togtistett . 15060 tegettegeg tgtegtgeog teettteggt ttettgteet tttogtittt tgggttteeg 15120 taatittitt gittiagitt ittiagiagi teggattiat aggigogiat tatigigita 15180 gittsettit tittligiet tittlegitge getegggitt tettatgitg gitaggitgg 15240 ttttgaattt tigatttogt gatttettig tttcggtttt ttaagetgit gggattatag 15300 sitteestte tietettiag toggitgita tittititti tittittoga toggitgita 15380 ttttttttt ttttttttt tttttttteg attgggtgtt atttttttt ttttttttt 15420 15480 15540 tttttttgat ategsetttt gtittettet tteggtiges gtgtagtgtt steettiest 15800 ttettgtage gtegattttt ttggtttagg tgattttttt gttttagttt tttaagtest 15660 15720 togtagtaga gatgaggttt ttttttatgt tgtttagggt ggttttgaat ttttgggttt 157B0 augigattit titattitag tittitiaaat tettaggatt ettatatett tegettatta 15840

[Drawing 45]

tatitegist tittittitaa tagagatggg gtittittat gitgittagg tiggiittas 15900 stilligagi tiaagiasti tiittittio ggittitias agigtigaga tistagaogi gogitatitt attiogitag gittitatit itsatatiti agittiaata iggitisati 16020 tgttgtggtt ttgttttcgt tttattaggg ttttttgtta oggatatatt gtttttttt 16080 igitigitti tetattaggg titigigiti attatitti tigittagag etgiligati 16140 titigattett acgitetetg gitagittit tittittitt attogggitt tagittamet 16200 gttatttttt ogggaaggtt titttgettt tittegtteg tagtgtiett tgtatattte 16260 ettattiett astatatese ettestitat tittittitt tittititt tiesestass 16320 gittiattit giogittagg tiggigigia giggigiaat tatogitgat igiagittig 16380 stittitage tittagiase tittitatit tagittitti stagitggas tistaggacg 16440 igigitatta tattiagite attititati tittgiagag stagggitti attetettes 16500 ttaggttggt tttaggtttt tgggtttagg tagtttttt gttttagttt tttatagtgt 18580 tgggettete ggtgtgeett attetettog gttmaggttg tttetttttt gogtmatett 16620 tttgagaatt tgtaatgatt taatttattg ggttatttgt toggttigta attitttatt 16680 genatetone titoetenen stossentta tetttetitt etitatitta etaffaffan 16740 tettttasat agtattiggt gietaetaga igittmeteo giattigtig saigesiget 16800 gtaggggasa gggaagtgaa aggaastasa gaagatggga ttaasgttig etgtigggaa 16860 gtgoggagna gtiggttgga ogtgegaggg ttttogoggg tggittitga ttgitatgit 16920 tgaggtgttg tgagttettt agggggateg gttttaagta ggaggtagea gtaggagttt 16990 egaggitigga egingenetg teetggageg estageteta tettigitta ggggaenetg 17040 ttesagaggg ggtagaggag tatgagtgag togggggttt ttatettttg ogggangggg 17100 sagittates gestoreses taggoriage starcestar storistita cettiniata 17160 ogtoggagtg agtattasta eagtittitt tittitisas attastagga gattaggatt 17220 togatggtgt togggattta tTTACGGGAT AGTATTTTT TGTAGGGAGG GGGAAGTTAC GOCGGAGGET CETAGGGGGC GGGGTTGAGT TAGGGGTATA GTTGTGTATA GTGGTTTAAB 17340 TTACGTGAGG AAGAATCGTT TAGTAATTAG TTATTTTTTT TTCGATTTTG TTTGTTAGGT 17400 TMTsgtatt gtagggatas attitggatt astagtittt aggasagge tegetgegg 17460 ggggttggtt ogagatogtt tatagoggtt agtttttttg tagttttasa gogggagtat 17520 agtaggites titgitggit tagggiegti tittiteggg agaggaggag tiatitggag 17580 titiatiter agittaugit gatagagoge ogggetatie getaggggaa tagattagga 17640 agtactitic gitragesar agtacticat gggggaaatt tittogtage oggaactica 17700 agggtttaag gaasattttt autagtitag tittttagag saggaggtat titttgtgta 17760 ggittatita tittagggogt tgagggatti attitagtit titaattigi ttogggggan 17820

[Drawing 46]

```
gaggaggagt tittaggtagg gittaggttit ggggtagggt taggittagt taggattata
                                                                      17880
gggaggagtt gtttaggggg atggggtttg gamaggtaga gggaattagg gamggagaga
                                                                      17940
tgggggttgt taggttogte agagettget attemggtte agitetaggo gtatttette
                                                                      18000
tigitiggee tatteaggit tittititit igstagiggi staagggagg gitaattiit
                                                                      18060
aggaggoggt tacgttgtts ttagtagtag gtttatgggg tggtagggtt atgggoggta
                                                                      18120
gazatagogt ttttagttig titgataggi iggogattit aggattitigg gittogiagg
                                                                      18180
ettigaties gegagisest timaggatet tiginagegg gegiggengg timattitit
                                                                      18240
tttteaggat tegogtgitt segstettig gagggggigg tegitteagt atgemtigit
                                                                      18300
ttittetteg tigggtagit gittgitase taggeogiet giaggittit gittogigig
                                                                     18360
gtitggatga igatettigg titetatigg gtateating atgg
                                                                      18404
```

[Drawing 47]

```
(a)
Wild type DNA
           5' -AGCTCGCGATGCCAGCTCGCTCG-3' sense strand
           3' -TCGAGCGCTACGGTCGAGCGAGC-5' antisense strand
(b)
Bisulfited
           5' -AGTTCGCGATGTTAGTTCGTTCG-3' sense strand
           3" -TTGAGCGCTATGGTTGAGCGAGC-5" antisense strend
(c)
FW primer
             5' -AGTTCGCGA
             5' -AGTTCGCGATGTTAGTTCGTTCG-3'
                           3'-TCAAGCAAGC-5'
                                                RV primer
(d)
             5' -AACTCGCGA
FW primer
             3' -TTGAGCGCTATGGTTGAGCGAGC-5' antisense strand
                           3'-TTGAGCGAGC-5' RV primer
```

[Drawing 48]

(a)

REP-S1: 5' -CAGGCCAGTGGAGTGGCAG-3'

(b)

REP-AS1: 5' -GAGGAGGTGCAGCTAGTCTG-3'

(c)

(#7441)

CAGGCCAGTGGAGTGGCAGCCCCAGAACTGGGACCACCGGGGGTGGTGA

REP-S1 Hpall

GGCGGCCCGGCACTGGGAGCTGCATCTGAGGCTTAGTCCCTGAGCTCTCT

Hpall

GCCTGCCCAGACTAGCTGCACCTCCTC (#7588)
REP-AS1

[Drawing 49]

(a)

REP-S2: 5' -CAAAGCACTGGCTTTGGAACC-3'

(b)

REP-AS2: 5'-ATCGAGTGAGTCCTGCTGGAT-3'

(c) (#6858)

<u>CAAAGCACTGGCTTTGGAACC</u>GGACTGTCTGGGTTTGAATCCTGGCACTG

REP-S2 Hipa II

CAGCTGACTGATGGACTCAGGCAATGCCTTAAACTCCCTGAGCCTC

AGGTTCCTTGTCTGTAAAATGATAAAGATAGCCCCTGTTTCATAGGGCTGT

GGTGAGAAACCAATCAGACAAGGCATGTGAACGCCATTATAGCACAGCG

CCCGGCATCCAGCAGGACTCACTCGAT (#7084)

Hpa II REP-AS2

[Drawing 50]

(a)

SHP1-PF1: 5'-TGTCTGGAGGCCACGGTCAATGA-3'

(b)

SHP1-PR1: 5' -GTTTGTATTCGGTTGTGTCATGCTC-3'

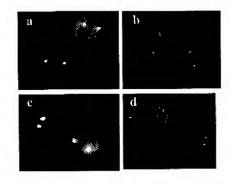
GGGTTCGCATGCGTGA (#7195)

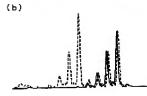
[Drawing 53]

(a)

FISH analysis of 1LTMot and NK-YS cells with chromosome
12 or SHP1 -specific probes

		positive signal No.(%)						
cells	probe	0	1	2	3	4	more	
ILTMot	Cb #12	1	1	97	1	O	0	
	SHP1	1	2	95	1	1	0	
NK-YS	Ch #12	0	0	99	i		0	
	SHP1	1	3	91	4	1	0	





Microsatellite marker	LOH					
D12S356	15/19 (79 %)					
D12S336	6/16 (38 %)					

CORRECTION OR AMENDMENT

[Kind of official gazette]Printing of amendment by the regulation of 2 of Article 17 of Patent Law

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C12N 15/09

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C12Q 1/42

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C12Q 1/44
G01N 27/447
G01N 33/53
G01N 33/566
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C12Q 1/02
C12Q 1/42
C12Q 1/44
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G01N 33/53 M
G01N 33/566
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G01N 27/26 301 A
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G01N 27/26 315 J

[Written amendment]
[Filing date]June 3, Heisei 16 (2004.6.3)
[Amendment 1]
[Document to be Amended]Specification
[Item(s) to be Amended]0046
[Method of Amendment]Change
[The contents of amendment]
[0046]

Therefore, in this invention, SHP1 gene expression can be judged using four-fold marker by the maximum called loss of ornamentation and mRNA of gene DNA, protein, and allele. namely,

one hematopoietic organ tumor cell called a SHP1 gene-expression fall -- since a specific phenomenon can be checked in <u>four steps</u>, hematopoietic organ tumor cells are detectable by very high singularity.

(19) 日本国特許庁(JP)

(12) 公 開 特 許 公 報(A)

(11)特許出願公開番号

特開2004-128 (P2004-128A)

(43) 公開日 平成16年1月8日 (2004.1.8)

(51) Int.C1. ⁷	F I			-	テーマコート	・ (参考)
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C12N 15/09	C12Q	1/02			4B063	
C12Q 1/02	C12Q	1/42				
C 1 2 Q 1/42	C12Q	1/44				
C12Q 1/44	GO1N	33/53	D			
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(21) 出願番号 (22) 出願日 (31) 優先權主張番号 (32) 優先日 (33) 優先權主張国	特願2002-331107 (P2002-331107) 平成14年11月14日 (2002.11.14) 特願2002-103495 (P2002-103495) 平成14年4月5日 (2002.4.5) 日本国 (JP)	(71) 出願人 (74) 代理人 (72) 発明者 (72) 発明者 F ターム (2	科琦0080034	版與事業町4g 市 課 課 課 課 明 中 市 市 市 市 市 市 市 市 目 (AA11 ()	4丁目1番 ;中1丁目4 屋敷2丁目	番2-304 2番58-1 CA20 HA09
					最	終頁に続く

(54) 【発明の名称】造血器腫瘍細胞検出方法および造血器腫瘍細胞検出キット

(57)【要約】

【課題】分子生物学的知見を利用して、造血器腫瘍細胞の高感度かつ高特異的に検出する造血器腫瘍細胞検出方法および検出キットを提供する。

【解決手段】造血器細胞を含む検体試料中に含まれる、造血器細胞に特異的なプロテインチロシンホスファターゼSHP1蛋白質を定量するとともに、上記検体試料から得られるSHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認する。これによって、一つの遺伝情報により造血器腫瘍細胞の有無を2段階で確認するため、非常に高い特異性で造血器腫瘍細胞を検出することができる。

【選択図】 なし

【特許請求の範囲】

【請求項1】

- (1) 造血器細胞を含む検体試料中に含まれる、造血器細胞に特異的なプロテインチロシンホスファターゼSHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認するSHP1遺伝子メチル化確認工程、
- (2) 上記検体試料から得られるSHP1蛋白質およびSHP1mRNAの少なくとも一方の発現量を定量するSHP1遺伝子産物定量工程、および、
- (3)上記検体試料に含まれるSHP1遺伝子の異型接合性喪失(LOH)の有無を確認するSHP1遺伝子LOH確認工程、
- の少なくとも何れかを含むことを特徴とする造血器腫瘍細胞検出方法。

【請求項2】

上記SHP1遺伝子メチル化確認工程には、

上記検体試料から得られた遺伝子試料を、シトシンを含む塩基配列を認識するメチル化感受性制限酵素で処理する遺伝子切断試行段階と、

上記メチル化感受性制限酵素で処理された遺伝子に対して、上記SHP1遺伝子の塩基配列中に含まれ、上記メチル化感受性制限酵素に認識切断される塩基配列を含む領域を増幅するプライマーを用いて、ポリメラーゼ連鎖反応法(PCR)を実施する遺伝子増幅試行段階と、

増幅された特定のサイズの遺伝子の量を確認する遺伝子増幅量確認段階とが含まれることを特徴とする請求項1に記載の造血器腫瘍細胞検出方法。

【請求項3】

上記プライマーが、さらに、配列番号1または2に示す塩基配列に含まれる部分塩基配列、またはこの部分塩基配列と相補性を有するポリヌクレオチドであることを特徴とする請求項2に記載の造血器腫瘍細胞検出方法。

【請求項4】

上記遺伝子増幅量確認段階では、電気泳動法を用いて特定サイズの遺伝子の量を確認することを特徴とする請求項2または3に記載の造血器腫瘍細胞検出方法。

【請求項5】

上記遺伝子切断試行段階では、制限酵素として、メチル化感受性制限酵素を用いることを特徴とする請求項2、3または4に記載の造血器腫瘍細胞検出方法。

【請求項6】

上記SHP1遺伝子メチル化確認工程には、

上記検体試料から得られる遺伝子試料を、重亜硫酸塩で処理する遺伝子修飾段階と、重亜硫酸塩で処理された遺伝子試料に含まれる、SHP1遺伝子の塩基配列中のメチル化シトシンの有無を判定するメチル化シトシン含有判定段階とが含まれることを特徴とする請求項1に記載の造血器腫瘍細胞検出方法。

【請求項7】

上記メチル化シトシン含有判定段階では、PCRによりメチル化シトシンを検出する方法、遺伝子の塩基配列の決定によりメチル化シトシンを検出する方法、またはメチル化シトシンを含む塩基配列を識別する方法のうち、少なくとも何れかが用いられることを特徴と 40 する請求項 6 に記載の造血器腫瘍細胞検出方法。

【請求項8】

上記遺伝子修飾段階では、重亜硫酸塩として、重亜硫酸ナトリウムが用いられることを特徴とする請求項 6 または 7 に記載の造血器腫瘍細胞検出方法。

【請求項9】

上記SHP1遺伝子産物定量工程では、SHP1蛋白質を抗原とするSHP1抗体を用いてSHP1蛋白質を定量することを特徴とする請求項1ないし8の何れか1項に記載の造血器腫瘍細胞検出方法。

【請求項10】

上記SHP1遺伝子産物定量工程では、酵素抗体法またはウエスタンブロッティング法に 50

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よりSHP1蛋白質が定量されることを特徴とする請求項9に記載の造血器腫瘍細胞検出方法。

【請求項11】

上記SHP1遺伝子産物定量工程では、配列番号3に示すSHP1遺伝子cDNAの塩基配列の全長またはその一部を検出するポリヌクレオチドを用いてSHP1遺伝子のmRNAの発現を検出することにより、SHP1mRNAを定量することを特徴とする請求項1ないし8の何れか1項に記載の造血器腫瘍細胞検出方法。

【請求項12】

上記SHP1遺伝子産物定量工程では、ノーザンブロッティング法、逆転写PCR法、リアルタイムPCR法、またはRNA in situハイブリダイゼーション法によりS 10 HP1遺伝子のmRNAの発現が検出されることを特徴とする請求項11に記載の造血器腫瘍細胞検出方法。

【請求項13】

異型接合性喪失の有無の確認は、上記SHP1遺伝子を挟み込む2つのマイクロサテライト・マーカーの少なくとも一方、または、上記SHP遺伝子中か、その近辺に存在する単一塩基多型のような遺伝子多型を、PCRを用いたフラグメント解析により実施されることを特徴とする請求項1ないし12の何れか1項に記載の造血器腫瘍細胞検出方法。

【請求項14】

造血器細胞を含む検体試料から造血器腫瘍細胞を検出するために用いられ、

- (1) 造血器細胞に特異的なプロテインチロシンホスファターゼSHP1蛋白質を抗原と 20 するSHP1抗体、および
- (2) シトシンを含む塩基配列を認識するメチル化感受性制限酵素と、

SHP1遺伝子の塩基配列中に含まれ、上記メチル化感受性制限酵素に認識される塩基配列を含む領域を増幅するPCR用のプライマーと、上記SHP1遺伝子のメチル化陽性及びメチル化陰性対照DNAとのうち、少なくとも一方を含むことを特徴とする造血器腫瘍細胞検出キット。

【請求項15】

造血器細胞を含む検体試料から造血器腫瘍細胞を検出するために用いられ、

- (1) 造血器細胞に特異的なプロテインチロシンホスファターゼSHP1蛋白質を抗原とするSHP1抗体、
- (2) 遺伝子処理レベルまで精製された重亜硫酸塩と、該重亜硫酸塩で処理された遺伝子試料に含まれるSHP1遺伝子の塩基配列中のシトシンの有無の判定用プライマー、および
- (3) 配列番号 3 に示す S H P 1 遺伝子 c D N A の塩基配列の全長またはその一部を検出する P C R 用のプライマーのうち、少なくとも何れか一つを含むことを特徴とする造血器腫瘍細胞検出キット。

【請求項16】

造血器細胞を含む検体試料から造血器腫瘍細胞を検出するために用いられ、

造血器細胞に特異的なプロテインチロシンホスファターゼSHP1遺伝子を挟み込む2つのマイクロサテライト・マーカーの少なくとも一方の全長またはその一部を検出するPC 40 R用のプライマーを含むことを特徴とする造血器腫瘍細胞検出キット。

【請求項17】

さらに、 P C R 用 試薬、 および、 制 限 酵素 反 応 用 試薬 の 少 な く と も 一 方 を 含 む こ と を 特 徴 と す る 請 求 項 1 4 、 1 5 ま た は 1 6 に 記 載 の 造 血 器 腫 瘍 細 胞 検 出 キ ッ ト 。

【発明の詳細な説明】

[0001]

【発明の属する技術分野】

本発明は、造血器腫瘍細胞検出方法および該検出方法に好適に用いられる造血器腫瘍細胞検出キットに関するものであり、特に、例えば、悪性リンパ腫や白血病等に特異的なプロテインチロシンホスファターゼSHP1遺伝子産物の発現減少あるいは消失またはこれを 50

コードする S H P 1 遺伝子のメチル化を検出することによって、 造血器 腫瘍 細胞を高感度 かつ 高特 異的に検出できる検出方法および検出キットに関するものである。

[0002]

【従来の技術】

ヒト(Homo sapiens)における悪性リンパ腫や白血病等の造血器腫瘍(血液系の腫瘍)には、難治性で極めて予後の悪いものから比較的予後のよいものまで様々な種類が知られている。この造血器腫瘍の治療には、各種化学療法や放射線療法、あるいは免疫療法といった種々の療法がすでに実用化されているが、このような治療の結果、ほぼ腫瘍細胞が退縮したとしても、わずかに腫瘍細胞が生存していれば造血器腫瘍の再発は免れない。

[0003]

上記造血器腫瘍の診断は、従来では、複数の診断手法を併用することにより総合的に実施されている。具体的には、末梢血や各種生検材料を用いて、組織染色や免疫染色等による形態学的な観察や組織学的な観察が実施されたり、さらには、種々の分子生物学的解析や染色体解析等も実施されたりしている。また、上記造血器腫瘍の診断では、判定までにかなりの時間を要する。

[0004]

【発明が解決しようとする課題】

上記従来の各診断手法は、それ単独では造血器腫瘍細胞を高感度、高特異的、かつ迅速に検出できるものではない。それゆえ、これら従来の診断手法では、複数を併用して総合的 20 に判断しなければ造血器腫瘍を診断することができない。

[0005]

つまり、従来の診断手法を使用する限り、複数の診断手法を併用しなければならないため、診断の煩雑化を招くだけでなく時間がかかり、造血器腫瘍細胞検出感度も特異性も高くないことから、医師の専門的な判断が診断に大きな比重を占めることになる。そのため、従来では、造血器腫瘍の診断技術は、実質的に医療現場での利用に限られており、各々の疾患者には対応できるが、集団検診による造血器腫瘍の早期発見・早期治療を目的としては利用されていない。

[0006]

造血器腫瘍細胞をより高感度かつ高特異的に実施するには、造血器腫瘍細胞に特異的であり、かつ広い範囲の造血器腫瘍に見られる感度の高いマーカーを用いることが考えられる。このようなマーカーを用いれば、造血器腫瘍の早期発見・診断を容易かつ迅速に実施することができ、医療上、悪性リンパ腫や白血病等の早期治療や再発予防に応用することが可能となるだけでなく、臨床検査産業や医薬品産業等にも利用可能な診断技術とすることができ、産業の発展に寄与することが可能となる。しかしながら、このようなマーカーは現在までのところ知られていない。

[0007]

本発明は上記課題に鑑みなされたものであって、その目的は、分子生物学的知見を利用して、迅速且つ簡便に造血器腫瘍細胞を高感度かつ高特異的に微量の患者検体から検出し造血器腫瘍の早期発見・診断および早期治療を容易にし、集団検診にも適用可能な造血器腫 40 瘍細胞検出方法および検出キットを提供することにある。

[0008]

【課題を解決するための手段】

本発明者らは、上記課題に鑑み鋭意検討した結果、広い範囲の悪性の造血器腫瘍では、プロテインチロシンホスファターゼSHP1蛋白質の発現抑制が極めて高頻度で見られ、しかも悪性度の高い造血器腫瘍において、上記SHP1蛋白質の発現抑制の傾向が強くなることを見出し、SHP1遺伝子産物およびこれをコードするSHP1遺伝子の双方をマーカーとして用いることで、造血器腫瘍細胞の高感度、高特異的、かつ短時間に検出でき、かつ産業上利用できる造血器腫瘍細胞検出技術を実現し得ることを見出し、本発明を完成するに至った。

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[0009]

すなわち、本発明にかかる造血器腫瘍細胞検出方法は、上記の課題を解決するために、(1)造血器細胞を含む検体試料中に含まれる、造血器細胞に特異的なプロテインチロシンホスファターゼSHP1蛋白質およびSHP1mRNAの少なくとも一方の発現量を定量するSHP1遺伝子産物定量工程、(2)上記検体試料から得られる、SHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認するSHP1遺伝子メチル化確認工程、および(3)上記検体試料に含まれるSHP1遺伝子の異型接合性喪失(LOH)の有無を確認するSHP1遺伝子LOH確認工程、の少なくとも一方を含むことを特徴としている。

[0010]

上記SHP1遺伝子産物の発現抑制は、悪性の造血器腫瘍細胞に極めて高頻度で見られるのに対し、正常な血液細胞にはこの現象が見られない。また、上記SHP1蛋白質の発現抑制は、SHP1遺伝子のメチル化によるものである。さらに、DNAメチル化によるSHP1遺伝子の転写抑制の前後には、SHP1遺伝子の一つの対立遺伝子が喪失している

[0011]

上記方法によれば、上記知見を利用して、検体試料から得られるSHP1遺伝子のメチル化を確認し、造血器腫瘍細胞の存在を検出することで、悪性の造血器腫瘍細胞の存在の有無をスクリーニングし、一方検体試料中のSHP1遺伝子産物、具体的にはSHP1蛋白質、またはSHP1mRNA、あるいはその両方の発現を定量する。

[0012]

すなわち、上記方法では、SHP1遺伝子の不活性化を、遺伝子DNAの修飾とmRNAと蛋白質と対立遺伝子の喪失という最大で四重のマーカーを用いて判定できることになる。すなわち、SHP1遺伝子の発現低下という一つの造血器腫瘍細胞特異的な現象を4段階で確認することができるため、非常に高い特異性で造血器腫瘍細胞を検出することができる。

[0013]

本発明にかかる造血器腫瘍細胞検出方法の好ましい一例としては、上記SHP1遺伝子メチル化確認工程に、上記検体試料から得られた遺伝子試料を、シトシンを含む塩基配列を認識するメチル化感受性制限酵素で処理する遺伝子切断試行段階と、上記メチル化感受性 30制限酵素で処理された遺伝子に対して、上記SHP1遺伝子の塩基配列中に含まれ、上記メチル化感受性制限酵素に認識切断される塩基配列を含む領域を増幅するプライマーを用いてPCR法を実施する遺伝子増幅試行段階と、増幅された特定のサイズの遺伝子の量を確認する遺伝子増幅量確認段階とが含まれる検出方法を挙げることができる。

[0014]

上記方法によれば、メチル化感受性制限酵素を用いて検体試料から得られた遺伝子試料に含まれるSHP1遺伝子の切断を試みることでメチル化の有無を区別し、さらにPCRを用いて増幅してから、得られる特定サイズのPCR産物の量を確認する。それゆえ、検体試料から微量のSHP1遺伝子さえ得られれば、SHP1遺伝子のメチル化を検出することができる。そのため、検体試料中に造血器腫瘍細胞がごく微量しか存在していなくても 40 高い検出感度で、しかも高特異的に造血器腫瘍細胞を検出することが可能となる。

[0015]

上記検出方法においては、上記プライマーが、さらに、配列番号 1 または 2 に示す塩基配列に含まれる部分塩基配列、またはこの部分塩基配列と相補性を有するポリヌクレオチドであることが好ましい。

[0016]

また、上記検出方法においては、上記遺伝子増幅量確認段階では、電気泳動法を用いて特定サイズの遺伝子の量を確認することが好ましい。

[0017]

さらに、上記検出方法においては、上記遺伝子切断試行段階では、メチル化感受性制限酵 50

素として、同一の塩基配列を認識するメチル化非感受性制限酵素が知られている制限酵素を用いることが好ましい。

[0018]

本発明にかかる造血器腫瘍細胞検出方法の好ましい他の一例としては、上記SHP1遺伝子メチル化確認工程に、上記検体試料から得られる遺伝子試料を、重亜硫酸塩で処理する遺伝子修飾段階と、重亜硫酸塩で処理された遺伝子試料に含まれる、SHP1遺伝子の塩基配列中のメチル化シトシンの有無を判定するメチル化シトシン含有判定段階とが含まれる検出方法を挙げることができる。

[0019]

上記方法によれば、重亜硫酸塩を用いて検体試料から得られた遺伝子試料を処理すると、塩基配列中のシトシンはウラシルに変換されるが、メチル化されたシトシンは変換されない。そのため、遺伝子修飾段階後のSHP1遺伝子の塩基配列中にシトシンが含まれるか否かを判定するのみで、SHP1遺伝子のメチル化を検出することができる。そのため、簡素なメカニズムで高特異的に造血器腫瘍細胞を検出することが可能となる。

[0020]

上記検出方法においては、上記メチル化シトシン含有判定段階では、PCRによりメチル化シトシンを検出する方法、遺伝子の塩基配列の決定によりメチル化シトシンを検出する方法、またはメチル化シトシンを含む塩基配列を識別する方法による遺伝子の処理のうち、少なくとも何れかが用いられても好ましい。

[0021]

上記方法によれば、少なくともPCRを用いることで、検体試料から微量のSHP1遺伝子さえ得られれば、SHP1遺伝子のメチル化を検出することができる。そのため、検体試料中に造血器腫瘍細胞がごく微量しか存在していなくても高い検出感度で高特異的に造血器腫瘍細胞を検出することが可能となる。

[0022]

上記検出方法においては、上記遺伝子修飾段階では、重亜硫酸塩として、重亜硫酸ナトリウムが用いられることが好ましい。また、上記遺伝子修飾段階では、重亜硫酸塩とともに尿素が併用されてもよい。

[0023]

本発明にかかる造血器腫瘍細胞検出方法においては、上記何れの例の検出方法であっても 30、上記SHP1遺伝子産物定量工程では、SHP1蛋白質を抗原とするSHP1抗体を用いてSHP1蛋白質を定量すると好ましい。具体的には、上記SHP1遺伝子産物定量工程では、酵素抗体法(免疫組織化学法、免疫細胞化学法、ELISA(enzyme-1inked immunosorbent assay)法)またはウエスタンプロッティング法によりSHP1蛋白質が定量されると好ましい。

[0024]

上記方法によれば、抗原抗体反応を利用してSHP1蛋白質を定量することになるので、 簡素なメカニズムで高特異的に造血器腫瘍細胞を検出することが可能となる。

[0025]

あるいは、本発明にかかる造血器腫瘍細胞検出方法においては、上記何れの例の検出方法 40であっても、上記SHP1遺伝子産物定量工程では、配列番号3に示すSHP1遺伝子 c DNAの塩基配列の全長またはその一部を検出するポリヌクレオチドを用いてSHP1遺伝子のmRNAの発現を検出することにより、SHP1mRNAを定量しても好ましい。具体的には、上記SHP1遺伝子産物定量工程では、ノーザンプロッティング法、逆転写PCR法、リアルタイム逆転写PCR法、またはRNA in situハイブリダイゼーション法によりSHP1遺伝子のmRNAの発現が検出されると好ましい。

[0026]

上記方法によれば、SHP1遺伝子産物としてSHP1遺伝子のmRNAによりSHP1 遺伝子産物を定量することになるので、SHP1遺伝子のcDNAと相同性を有するオリ ゴペプタイドをプローブやプライマーとして利用することで、簡素なメカニズムで高特異 50 的かつ高感度に造血器腫瘍細胞を検出することが可能となる。

[0027]

本発明にかかる造血器腫瘍細胞検出方法の好ましいさらに他の一例としては、異型接合性 要失の有無の確認は、上記SHP1遺伝子を挟み込む2つのマイクロサテライト・マーカ 一の少なくとも一方、または、上記SHP1遺伝子中か、その近辺に存在する単一塩基多 型のような遺伝子多型を、PCRを用いたフラグメント解析することにより実施される方 法を挙げることができる。このとき用いられる検体試料は、造血器細胞を含む検体試料で あればよい。また、対照としては、血液学的完全寛解後に得られる検体を用いてもよいし 、他の正常組織細胞を用いてもよい。

[0028]

上記の方法によれば、マイクロサテライト・マーカーまたは単一塩基多型(SNP)等の遺伝子多型の異型接合性喪失をPCRにより確認することによって、SHP1遺伝子の異型接合性喪失を確認しているので、簡素なメカニズムでより確実に造血器腫瘍細胞を検出することが可能となる。

[0029]

本発明にかかる造血器腫瘍細胞検出キットの好ましい一例としては、造血器細胞を含む検体試料から造血器腫瘍細胞を検出するために用いられ、(1)造血器細胞に特異的なプロテインチロシンホスファターゼSHP1蛋白質を抗原とするSHP1抗体、および、(2)シトシンを含む塩基配列を認識するメチル化感受性制限酵素と、SHP1遺伝子の塩基配列中に含まれ、上記メチル化感受性制限酵素に認識切断される塩基配列を含む領域を増 20幅するPCR用のプライマーと、上記SHP1遺伝子のメチル化陽性及びメチル化陰性対照DNAとのうち、少なくとも一方を含む構成を挙げることができる。

[0030]

あるいは、本発明にかかる造血器腫瘍細胞検出キットの好ましい他の一例としては、造血器細胞を含む検体試料から造血器腫瘍細胞を検出するために用いられ、(1)上記SHP1抗体、および(2)遺伝子処理レベルまで精製された重亜硫酸塩と、該重亜硫酸塩で処理された遺伝子試料に含まれるSHP1遺伝子の塩基配列中のシトシンの有無の判定用プライマー、および、(3)配列番号3に示すSHP1遺伝子cDNAの塩基配列の全長またはその一部と相同性を持つPCR用のプライマーのうち、少なくとも何れか一つを含む構成を挙げることができる。

[0031]

さらには、本発明にかかる造血器腫瘍細胞検出キットの好ましいさらに他の一例としては、造血器細胞を含む検体試料から造血器腫瘍細胞を検出するために用いられ、造血器細胞に特異的なプロテインチロシンホスファターゼSHP1遺伝子を挟み込む2つのマイクロサテライト・マーカーの少なくとも一方の全長またはその一部を検出するPCR用のプライマーを含む構成を挙げることができる。

[0032]

上記造血器腫瘍細胞検出キットにおいては、さらに、PCR用試薬、および、制限酵素反応用試薬の少なくとも一方を含むことが好ましい。

[0033]

上記何れの構成であっても、前述した造血器腫瘍細胞検出方法を実施するために好ましい 薬剤や標本等が含まれている。そのため、上記検出キットを用いることで、本発明にかか る造血器腫瘍細胞検出方法を容易かつ迅速に実施することができ、本発明を臨床検査産業 や医薬品産業等の産業レベルで利用することが可能となる。

[0034]

【発明の実施の形態】

〔実施の形態1〕

本発明における実施の一形態について図 1 ないし図 2 4 に基づいて説明すれば以下の通りである。なお、本発明はこれに限定されるものではない。

[0035]

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本発明は、造血器細胞を含む検体試料中に含まれる、造血器細胞に特異的なプロモーターからのプロテインチロシンホスファターゼSHP1遺伝子産物すなわちSHP1蛋白質とmRNAとを定量するとともに、上記検体試料から得られる、SHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認することで、上記検体試料中から造血器腫瘍細胞を検出する技術である。

[0036]

本発明で、造血器腫瘍細胞を検出するためのマーカーとして用いられるSHP1遺伝子は、染色体12p13に存在し、図1~図10および配列番号1に示す塩基配列をゲノムDNA(ワイルドタイプ)のセンス鎖とし、図11~図20および配列番号2に示す塩基配列をアンチセンス鎖とする16のエキソン(図および配列表中大文字で示す領域)を有す 10る遺伝子である。そのcDNAは、図21および配列番号3に示す塩基配列を有する約1.8kbのサイズを有している。なお、SHP1遺伝子はSH-PTP1,PTP1C、HCP、HCPH、PTPN6、HPTP1C、SHP-1Lと同一の遺伝子である。

[0037]

上記SHP1遺伝子にコードされているSHP1蛋白質は、分子量68kDで、各種造血器細胞に特異的なプロテインチロシンホスファターゼ(PTPase)であり、図22に示すように、N末端側にタンデム構造となる2つのSH2(Src homologydomain 2)領域(270アミノ酸残基)と、246アミノ酸残基のPTPaseドメインと、93アミノ酸残基のC末端側領域とを有する構造となっている。また、図23および配列番号4に示すアミノ酸配列を有している。

[0038]

ヒトの造血器腫瘍、例えば悪性リンパ腫や白血病では、多くの種類で90%以上の高い頻度でSHP1蛋白質の強い発現抑制が見られる(例えば、American Journal of Pathology, Vol.159, No.4, October 2001:1495-1505等参照)。このように悪性の造血器腫瘍細胞では、上記SHP1蛋白質の発現抑制が極めて高頻度で見られるのに対し、正常な血液細胞にはこの現象が見られない。

[0039]

本発明者らは、上記SHP1蛋白質の発現抑制が、上記SHP1遺伝子がメチル化されることによる転写異常を原因とすることを独自に見出した。

[0040]

例えば、図24に示すように、図1~図10および配列番号1に示すゲノムDNA(ワイルドタイプ)のセンス鎖(181塩基~2160塩基まで例示)において、1001塩基~1163塩基のエキソン(図中大文字)の前にプロモーター領域が存在するが、この近傍にシトシン(C)とグアニン(G)とが並ぶCG配列が多く存在しCpG島(CpGisland)を形成している(図24中ではCG配列を太字の網掛けで示す)。正常な造血器細胞では、このCpG島のシトシンはメチル化されていないが、例えば悪性のリンパ腫細胞では、上記CG配列のシトシンの多くがメチル化されている。勿論、このCG配列におけるシトシンのメチル化はセンス鎖のみならずアンチセンス鎖にも同じように生じる。

[0041]

上記 C p G 島における C G 配列の高度なメチル化は、 S H P 1 遺伝子の D N A から m R N A の 転写を 阻害し、その結果、 S H P 1 蛋白質の生産が抑制される。この現象は、上述したように 造血器 腫瘍細胞では極めて高頻度に見られる。しかも、各種 造血器 腫瘍 患者の完全 寛解期には、 S H P 1 遺伝子における D N A のメチル化が完全に消失し、 分子生物学上の知見と 臨床上の知見との間に非常に高い相関関係が見られる。それゆえ、メチル化による S H P 1 遺伝子の発現抑制が、造血器 腫瘍細胞の発症機構の中で重要な役割を果たしていることが推測される。そこで本発明では、上記 S H P 1 遺伝子の発現抑制という現象を、造血器 腫瘍細胞のマーカーとして利用する。

[0042]

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さらに、本発明者らは、悪性リンパ腫や白血病等の疾患が発症する際に、上述した、DNAメチル化によってSHP1遺伝子の転写抑制が生じる前後に、SHP1遺伝子の一つの対立遺伝子が喪失することも独自に見出した。そこで、SHP1遺伝子の異型接合性喪失を確認することによって、SHP1遺伝子の対立遺伝子の喪失を確認することが可能となる。それゆえ、SHP1遺伝子の異型接合性喪失も造血器腫瘍細胞のマーカーとして利用することができる。

[0043]

悪性リンパ腫や白血病において、SHP1遺伝子には、高頻度のDNAメチル化、高頻度の異型接合性喪失、SHP1遺伝子の発現の低下または消失が検出され、さらには、外来SHP1遺伝子導入が血球系の細胞の増殖を抑制する傾向にある。これにより、SHP1 10遺伝子が癌抑制遺伝子の一つであることが強く示唆される。

[0044]

そこで、本発明では、SHP1遺伝子メチル化確認工程で、上記検体試料から得られるSHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認し、SHP1遺伝子産物定量工程にて、造血器細胞を含む検体試料中に含まれるSHP1蛋白質およびmRNAの少なくとも一方を定量し、さらに、SHP1遺伝子LOH確認工程で、SHP1遺伝子の異型接合性喪失を確認するという三つの工程を利用する。これら工程は単独で用いられても良いし、双方ともに用いられても良い。さらに、SHP1遺伝子産物定量工程では、SHP1蛋白質のみ定量されても良いし、SHP1mRNAのみ検出されても良いし、双方ともに検出されてもよい。

[0045]

これによって、例えば、まず、検体試料中のSHP1遺伝子のメチル化を検出することでスクリーニングし、その後、検体試料のSHP1遺伝子産物の発現をSHP1mRNAおよびSHP1蛋白質の少なくとも一方で定量することで、悪性の造血器腫瘍細胞の有無を確認することで造血器腫瘍細胞の存在を確定するという検出プロセスを実施することができる。

[0046]

したがって、本発明では、SHP1遺伝子の発現を、遺伝子DNAの修飾とmRNAと蛋白質と対立遺伝子の喪失という最大で四重のマーカーを用いて判定できることになる。すなわち、SHP1遺伝子の発現低下という一つの造血器腫瘍細胞特異的な現象を3段階で30確認することができるため、非常に高い特異性で造血器腫瘍細胞を検出することができる

[0047]

また、上述したように、本発明におけるSHP1遺伝子を導入することで、血球系の細胞の増殖を抑制する傾向にあることも確認されている。それゆえ、SHP1遺伝子は、遺伝子治療に用いることも可能であり、例えば、腫瘍細胞にSHP1遺伝子の発現ベクターをトランスフェクトすることにより、腫瘍細胞の増殖を抑制することが期待できる。

[0048]

本発明で用いられる検体試料は、末梢血あるいは骨髄液等の造血器細胞を含む検体試料であればどのような検体試料であっても特に限定されるものではない。本発明における造血 40 器細胞とは、各種血液細胞を含むが、特に好ましくは各種白血球が挙げられる。より具体的には、リンパ球(T細胞・B細胞)、顆粒球(好中球、好酸球、好塩基球)、単球並びにマクロファージ、マスト細胞、ナチュラルキラー細胞等を挙げることができる。あるいは造血幹細胞やリンパ球幹細胞であってもよい。

[0049]

したがって、本発明で用いられる検体試料には、上記造血器細胞が含まれている血液や骨髄液あるいは体液等をヒトから採取し、これをそのまま検体試料として用いてもよいし、採取した血液や体液に対して従来公知の処理を施すことによって、分子生物学的な分析を実施し易い分析用検体試料としてもよい。

[0050]

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本発明が適用可能な造血器腫瘍としては、具体的には、例えば、慢性骨髄性白血病、フィ ラデルフィア染色体ポジティブ (+ (9 ; 2 2) (q q 3 4 ; q 1 1) , B C R / A B L)慢性骨髓性白血病、慢性好中球白血病、慢性好酸球白血病/高好酸球症候群、慢性突発 性骨髄繊維症、真性多血症、本態性血小板増加症、その他分類できない骨髄増殖性疾患等 の各種骨髄増殖性疾患;

慢性骨髄性単球白血病、非定型慢性骨髄性白血病、幼年性骨髄性単球白血病等の骨髄異型 性/骨髓增殖性疾患;

環状鉄芽球を伴う難治性貧血、環状鉄芽球を伴わない難治性貧血、多系列異形成を伴う難 治性血球減少症(骨髄異型性症候群)、過剰芽球5Q-症候群を伴う難治性貧血(骨髄異 型性症候群)、その他分類できない骨髄異型性症候群等の骨髄異型性症候群;

再発性細胞遺伝学的転座を伴う急性骨髄性白血病(AML)(例えば、+(8;21)(q 2 2 ; q 2 2) を伴う A M L 、 A M L 1 (C B F - α) / E T O 、 急性前骨髄性白血病 (+ (15;17) (q22;q11-12) を伴うAMLおよびその変形、PML/R $AR-\alpha$))、異常な骨髄好酸球(inv(16)(p13q22)あるいは+(16; 16) (p13; q11)、CBF β / MYH11X) を伴うAML、11q23 (ML L)異常を伴うAML、前骨髄異型性症候群を伴いかつ多系列異形成を伴うAML、前骨 髄異型性症候群を伴いかつ多系列異形成を伴わないAML、治療に関係するAMLおよび 骨髄異型性症候群(アルキル化剤に関係する治療、エピポドフィロトキシンに関係する治 療、あるいはその他のタイプの治療)、他に部門に属さないAML(低分化型、成熟を伴 わないもの、成熟を伴うもの、急性骨髄性単球白血病、急性単球白血病、急性赤芽球白血 20 病、急性巨核球白血病、急性好塩基球白血病、骨髄繊維症を伴う急性汎骨髄過剰増殖症)

前駆体B細胞性腫瘍(前駆体B-リンパ芽球性白血病/リンパ腫(前駆体B細胞急性リン パ芽球性白血病)、成熟(末梢)B細胞性腫瘍(B細胞慢性リンパ球性白血病/小リンパ 球性リンパ腫、B細胞前リンパ球性白血病、リンパ形質細胞性リンパ腫、脾辺縁領域B細 胞リンパ腫(+/-絨毛リンパ球)、毛状細胞白血病、形質細胞性骨髄腫(形質細胞腫) MALT型節外辺縁型B細胞リンパ腫、節性辺縁型B細胞リンパ腫(+/-細 胞) 、 濾 胞 性 リ ン パ 腫 、 マ ン ト ル 細 胞 リ ン パ 腫 、 び ま ん 性 大 型 B 細 胞 リ ン パ 腫 (縦 隔 大 細胞B細胞リンパ腫、原発性滲出リンパ腫)、Burkitt リンパ腫/Burkit t 細胞白血病) 等のB細胞性腫瘍;

、急性二形質性白血病等の急性骨髄性白血病(AML);

前駆体T細胞性腫瘍(前駆体T-リンパ芽球性白血病/リンパ腫(前駆体T細胞急性リン パ芽球性白血病)、成熟(末梢)T細胞性腫瘍(T細胞前リンパ球性白血病、T細胞顆粒 リンパ球白血病、侵攻型NK細胞白血病、成人T細胞リンパ腫・白血病(HTLV1+) 、鼻型節外性NK/T細胞リンパ腫、腸管症型T細胞リンパ腫、肝脾型ү-δT細胞リン パ腫、皮下蜂窩織炎様T細胞リンパ腫、菌状息肉腫/Sezary症候群、退形成性大型 細胞リンパ腫(T/ヌル細胞、原発性皮膚未分化型)、他に部門に属さない末梢T細胞リ ンパ腫、血管免疫芽球T細胞リンパ腫)等のT細胞およびNK細胞性腫瘍;

節 性 リンパ 球 優 勢 ホ ジ キ ン リ ンパ 腫 、 古 典 的 ホ ジ キ ン リ ンパ 腫 (結 節 硬 化 ホ ジ キ ン リ ン パ 腫 (等級1および2)、リンパ球リッチ古典的ホジキンリンパ腫、混合細胞型ホジキンリ ンパ腫、リンパ球枯渇ホジキンリンパ腫)等のホジキンリンパ腫(ホジキン病); 等を挙げることができるが、特に限定されるものではない。

[0051]

本発明におけるSHP1遺伝子産物定量工程は、検体試料中のSHP1蛋白質およびSH P1mRNAの少なくとも一方を定量できる方法であれば特に限定されるものではないが 、具体的には、SHP1蛋白質を抗原とするSHP1抗体を用いてSHP1蛋白質を定量 する方法(蛋白質定量法)か、SHP1遺伝子のmRNAの発現を検出することにより、 SHP1mRNAを定量する方法(mRNA定量法)を好適に用いることができる。

まず、上記蛋白質定量法のより具体的な手法としては、SHP1抗体を用いたウエスタン ブロッティング法または酵素抗体法(Immunochemistry)(免疫組織化学 50

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法、免疫細胞化学法、ELISA (enzyme-linked immunosorbent assay) 法) を挙げることができる。

[0053]

上記蛋白質定量法で用いられるSHP1抗体は、図22、図23および配列番号4に示す構造を有するSHP1蛋白質の少なくとも一部の構造を抗原決定基として認識し、SHP1蛋白質を免疫学的に確実に検出できる抗体であれば特に限定されるものではなく、ポリクローナル抗体であってもよいし、モノクローナル抗体であってもよい。

[0054]

上記 S H P 1 抗体は、従来公知の方法で製造してもよいし、市販の S H P 1 抗体を用いてもよい。 S H P 1 抗体の製造方法としては、例えば、モノクローナル抗体であれば、 S H 10 P 1 蛋白質で免疫したマウス pp 臓リンパ球とマウスの骨髄細胞とを融合させてなるハイブリドーマにより産生する手法が挙げられる。また、上記 S H P 1 抗体がポリクローナル抗体であれば、 S H P 1 蛋白質で免疫したウサギの免疫血清から精製する手法が挙げられる。また、市販の S H P 1 抗体としては、 # S H - P T P 1 (D - 1 1): s c 7 2 8 9 および # S H - P T P 1 (C - 1 9): s c 2 8 7 (S a n t a C r u z Biotechnology I n c . 製)、 # a n t i S H P T P (0 6 1 1 7) および # a n t i m o u s e S H P T P (0 5 2 8 1))

(Upstate Biotechnology Inc. 製)等が挙げられる。

[0055]

上記SHP1抗体を用いた酵素抗体法(免疫組織化学法、免疫細胞化学法、ELISA法 20)は、従来公知の方法(例えば、『酵素抗体法』渡辺慶一・中根一穂編集、学際企画出版(昭和61年)や、Brown R.W. et al: Modern Pathol. 199;8(5)515-20(1995)等の文献に開示されている方法)を好適に用いることができ、その具体的な工程や試薬類、条件等は特に限定されるものではない

[0056]

同様に、上記SHP1抗体を用いたウエスタンプロッティング法も、従来公知の方法(例えば、『実験操作ブロッティング法』日野嘉幸他編、ソフトサイエンス社(昭和62年)や、Towbin H. et al: Proc.Natl.Acad.Sci.USA76,4350,(1979) 等の文献に開示されている方法)を好適に用いること 30ができ、その具体的な工程や試薬類、条件等は特に限定されるものではない。

[0057]

上記蛋白質定量法を用いることで、抗原抗体反応を利用してSHP1蛋白質を定量することになる。そのため、簡素なメカニズムで高特異的に造血器腫瘍細胞を検出することが可能となる。

[0058]

次に、上記mRNA定量法のより具体的な手法としては、配列番号3(図21参照)に示すSHP1遺伝子cDNAの塩基配列の全長またはその一部と相同性を有するポリヌクレオチドを用いてSHP1遺伝子のmRNAの発現を検出する方法が挙げられ、より具体的には、ノーザンブロッティング法、逆転写ポリメラーゼ連鎖反応法(RT-PCR)、リ 40アルタイム逆転写ポリメラーゼ連鎖反応法(real time RT-PCR)、またはRNA in situハイブリダイゼーションを挙げることができる。

[0059]

上記ノーザンブロッティング法、RT-PCR、real time RT-PCR、およびRNA in situハイブリダイゼーションの何れの方法も従来公知の方法(例えば、"Molecular cloning" a laboratory manual, Sambrook J., Russell DW., Cold Spring Harbor Lab Press. (2001) や、"Current protocols in molecular biology" edited by Ausubel FM et al., John Wiley & Sons Inc. (250

001) 等の文献に開示されている方法)を好適に用いることができ、その具体的な工程や試薬類、条件等は特に限定されるものではない。

[0060]

上記ノーザンブロッティング法やRNA in situ ハイブリダイゼーションでは、原理的には、配列番号3に示すSHP1遺伝子のcDNAの全長あるいはその一部をプロープとして用いることができる。また、RT-PCRやreal time RT-PCRでも、原理的には、配列番号3に示すSHP1遺伝子のcDNAの一部と相同性を有するオリゴヌクレオチドをプライマーとして用いることができる。具体的には、例えば、後述する実施例3や実施例4に示すプライマーペアを用いることができる。

[0061]

それゆえ、mRNA定量法では、配列番号3に示すSHP1遺伝子cDNAの塩基配列の全長またはその一部と相同性を有するポリヌクレオチドを用いてSHP1遺伝子のmRNAの発現を検出すればよい。

[0062]

上記mRNA定量法を用いることで、SHP1遺伝子の転写産物であるSHP1mRNA を定量することになるので、SHP1遺伝子のcDNAと相同性を有するポリヌクレオチドをプローブやプライマーとして利用することで、簡素なメカニズムで迅速、高特異的かつ高感度に造血器腫瘍細胞を検出することが可能となる。

[0063]

本発明におけるSHP1遺伝子メチル化確認工程は、検体試料から得られるSHP1遺伝 20子の塩基配列中に含まれるCpG島のメチル化を確認できる方法であれば特に限定されるものではないが、本実施の形態では、例えば、遺伝子切断試行段階と、遺伝子増幅試行段階と、遺伝子増幅量確認段階とを含むメチル化感受性制限酵素を利用した方法(以下、説明の便宜上、制限酵素確認法と称する)を好適に用いることができる。

[0064]

本実施の形態で用いられるメチル化感受性制限酵素とは、二本鎖 DNAにおいて認識対象となる塩基配列にシトシンを含んでおり、かつ、この塩基配列中のシトシンがメチル化された場合には、該塩基配列の二本鎖 DNAを切断できない制限酵素であれば特に限定されるものではない。

[0065]

上記メチル化感受性制限酵素としては、具体的には、例えば、HpaII、EagIまたはNaeI等を挙げることができる。中でも、HpaIIをより好ましく用いることができる。HpaIIは、CCGGの塩基配列を認識して二本鎖DNA切断するエンドヌクレアーゼであるが、同じ塩基配列を認識して二本鎖DNAを切断する制限酵素として、MspIが知られている。

[0066]

上述したように、HpaIIはメチル化されたCCGGの塩基配列の二本鎖DNAを切断できないが、MspIはメチル化の有無に関わらずCCGGの塩基配列を認識して二本鎖DNAを切断することが可能である。すなわち、MspIはメチル化非感受性制限酵素である。それゆえ、HspIIとMspIとを併用することで、後述するように、検体試料 40中のSHP1遺伝子の切断を確実に確認するためのコントロールとして利用することが可能になり、本実施の形態における制限酵素確認法の信頼性をより一層向上させることができる。

[0067]

このように、本実施の形態における制限酵素確認法では、使用するメチル化感受性制限酵素と同じ塩基配列を認識するメチル化非感受性制限酵素をコントロールとして使用することが好ましい。勿論、メチル化感受性およびメチル化非感受性制限酵素の組み合わせは上記 HspII・MspIに限定されるものではないことは言うまでもない。

[0068]

次に、本実施の形態におけるSHP1遺伝子メチル化確認工程、すなわち制限酵素確認法 50

によるSHP1遺伝子のメチル化の確認について具体的に説明する。

[0069]

まず、遺伝子切断試行段階として、造血器細胞を含む前記検体試料から得られた遺伝子試料を、シトシンを含む塩基配列を認識する上記メチル化感受性制限酵素で処理する。この段階では、メチル化感受性制限酵素の処理により遺伝子試料中に含まれるSHP1遺伝子の切断を試みる。すなわち、前記検体試料中に含まれる造血器細胞が正常な細胞のみであれば、SHP1遺伝子は切断されるが、造血器腫瘍細胞が含まれていれば、SHP1遺伝子はCG配列がメチル化されているため切断されない。

[0070]

前記検体試料から遺伝子試料を調製する方法は従来公知の方法を用いることができ特に限定されるものではない。また、調製された遺伝子試料は、SHP1遺伝子を含んでいればよく、制限酵素処理やPCR等を阻害しない限り他の成分が含まれていても良い。それゆえ、前記検体試料中に含まれる造血器細胞やその他の細胞から抽出される各種DNAやRNAの混合物であればよい。また、メチル化感受性制限酵素による処理についても特に限定されるものではなく、該メチル化感受性制限酵素の種類や調製された遺伝子試料の状態等に応じて、適宜条件等を設定すればよい。

[0071]

次に、遺伝子増幅試行段階として、上記メチル化感受性制限酵素で処理された遺伝子試料に対して、上記SHP1遺伝子の塩基配列中に含まれ、上記メチル化感受性制限酵素に認識切断される塩基配列を含む領域を増幅するプライマーを用いて、PCRを実施する。こ 20の段階では、メチル化感受性制限酵素で処理した制限酵素処理物を、上記プライマーを用いてPCR処理することにより、SHP1遺伝子のみの増幅を試みる。正常なSHP1遺伝子のみであれば、プライマーペアに挟まれる領域が切断されているためSHP1遺伝子は増幅できないが、メチル化されているSHP1遺伝子が含まれていれば、上記プライマーペアに挟まれる領域は切断されていないためSHP1遺伝子が増幅される。

[0072]

上記遺伝子増幅試行段階で用いられる上記プライマーとしては、メチル化感受性制限酵素に認識される塩基配列を含む領域を増幅するポリヌクレオチドであればよい。それゆえプライマーの設計条件等についても特に限定されるものではない。基本的には、本実施の形態で用いられるプライマーペアは、メチル化感受性制限酵素に認識される上記塩基配列を 30 含む領域の少なくとも外側に位置し、配列番号1または2(図1~図10および図11~図20参照)に示すSHP1遺伝子の塩基配列に含まれる部分塩基配列、またはこの部分塩基配列と相補性を有するポリヌクレオチドであればよく、その場所やサイズ等については特に限定されるものではない。

[0073]

次に、遺伝子増幅量確認段階として、増幅された遺伝子の量を確認する。この段階では、 SHP1遺伝子が増幅されたか否かを確認する。SHP1遺伝子が増幅されれば、元の検 体試料中に造血器腫瘍細胞が含まれていることになる。

[0074]

上記遺伝子増幅量確認段階で用いられるSHP1遺伝子の有無の確認方法としては特に限 4 定されるものではないが、電気泳動法を用いてマーカーと比較することにより遺伝子の増幅量を確認する手法が最も一般的で確立された手法であるため好ましく用いることができる。また、電気泳動後に得られたDNAバンドをメンプレンにブロッティングして検出してもよい。

[0075]

上記遺伝子増幅量確認段階で用いられるSHP1遺伝子の有無の確認方法としては特に限定されるものではないが、検体試料と同時にメチル化陽性およびメチル化陰性対照DNAを用いて反応を行った後電気泳動法を用いて遺伝子の増幅量を確認する手法が最も一般的で確立された手法であるため好ましく用いることができる。また、電気泳動後に得られたDNAバンドをメンブレンにブロッティングして検出してもよい。

[0076]

上記SHP1遺伝子のメチル化陽性およびメチル化陰性対照DNAは、SHP1遺伝子を用いたものであればよく、特に限定されるものではない。具体的には、メチル化感受性制限酵素またはメチル化非感受性制限酵素により処理することで得られる、増幅量を比較できる程度の濃度を有するDNA溶液を挙げることができる。

[0.077]

さらに、制限酵素確認法によるSHP1遺伝子メチル化確認工程では、コントロールとして、メチル化感受性制限酵素による処理と並行して、同一の検体試料をメチル化非感受性制限酵素で処理して、それを遺伝子増幅量確認段階で確認すると好ましい。すなわち、上記遺伝子切断試行段階では、メチル化感受性制限酵素として、同一の塩基配列を認識する 10メチル化非感受制限酵素が知られている制限酵素を用いることが非常に好ましい。これによって、制限酵素確認法によるSHP1遺伝子のメチル化の確実性を高めることができる

[0078]

本発明におけるSHP1サテライトLOH確認工程は、造血器細胞を含む検体試料において、この検体試料に含まれるSHP1遺伝子の異型接合性喪失(Lossof heterozygosity,LOHと略す)の有無を確認することができる方法であれば特に限定されるものではないが、具体的には、SHP1遺伝子を挟み込むマイクロサテライト・マーカー、または、上記SHP遺伝子中か、その近辺に存在する単一塩基多型(single nucleotide polymorphism,SNP)のような遺伝子多 20型(polymorphism)について、PCRを用いたフラグメント解析によってLOHを確認する方法を好適に用いることができる。

[0079]

上記SHP1遺伝子の両側に存在するマイクロサテライト・マーカーや、SHP1遺伝子中またはその近辺に存在する遺伝子多型については、特に限定されるものではなく、どのようなマーカーを用いてもよいが、具体的には、例えば、D12S336マーカーおよびD12S356マーカーを挙げることができる。これらマーカーの塩基配列は、インターネット・ゲノム・データベース(URL:http://gdbwww.gdb.org./)から得られる。これらマーカーのうち、D12S356マーカーはテロメア側に存在し、SHP1遺伝子から約4.4cMの距離にある。一方、D12S356マーカーは 30セントロメア側に存在し、SHP1遺伝子から約2.4cMの距離にある。

[0080]

検体試料におけるSHP1遺伝子のLOH(異型接合性喪失)の確認に際しては、SHP1サテライトLOH確認工程で用いられる検体試料は、造血器細胞を含む検体試料となっていればよい。また、LOHの具体的な方法は特に限定されるものではないが、後述する実施例6に示すように、PCR反応によって上記各マーカーの少なくとも一方の全長またはその一部を検出するマイクロサテライト解析を行えばよい。このときのPCR反応他の条件も特に限定されるものではなく、PCR用のプライマーとしては、例えば、D12S336マーカーまたはD12S356マーカーの少なくとも一部を検出できるようなプライマーであればよく、その他の条件についても適切な条件を適宜設定すればよい。

[0081]

本発明におけるSHP1サテライトLOH確認工程で用いられる検体試料は、造血器細胞を含む検体試料であれば特に限定されるものではない。また、対照として用いる検体試料も特に限定されるものではなく、血液学的完全寛解後に得られる検体を用いてもよいし、他の正常組織細胞を用いてもよい。

[0082]

このように、マイクロサテライト・マーカーやSNP等の遺伝子多型を利用してSHP1 遺伝子のLOHを確認することで、簡素なメカニズムでより確実に造血器腫瘍細胞を検出することが可能となる。

[0083]

なお、本実施の形態では、SHP1遺伝子のLOHを、マイクロサテライト・マーカーや遺伝子多型を利用して確認した例を挙げているが、本発明はこれに限定されるものではなく、SHP1遺伝子のLOHが確認できる方法であればどのような方法でもよいことは言うまでもない。

[0084]

次に、本実施の形態にかかる検出方法の好ましい一例について、より具体的に説明する。

[0085]

まず、SHP1遺伝子産物定量工程により、前述した手法を用いて検体試料中に含まれるSHP1蛋白質およびSHP1mRNAの少なくとも一方を定量する。このプロセスで定量されたSHP1蛋白質が、標準よりも大幅に減少していたり、ほとんどSHP1遺伝子 10産物が発現していなかったりした場合には、検体試料中に造血器腫瘍細胞が含まれている可能性が高くなる。

[0086]

次に、SHP1遺伝子メチル化確認工程で、前記制限酵素確認法により、検体試料から調製した遺伝子試料中のSHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認する。以下の説明では、メチル化感受性制限酵素として前記HpaIIを用いた例を挙げる。HpaIIは、前述したようにCCGGの塩基配列を認識するが、同じ塩基配列を、メチル化非感受性制限酵素MspIも認識するため、好ましく用いられる。

[0087]

そこで、遺伝子切断試行段階では、上記検体試料から得られた遺伝子試料を、HpaII 20で処理する。同時に、同一の遺伝子試料をMspIで処理すると好ましい。これによって、CCGG塩基配列が切断されるというポジティブコントロールを得ることができる。

[0088]

次に、遺伝子増幅試行段階に移行するが、このステップでは、先に、SHP1遺伝子の塩基配列(配列番号1および2、図1~図10および図11~図20参照)から、HpaII/MspIの認識部位(CCGG)を挟んでPCR用のプライマーを設定する。具体的には、例えば、後述する実施例1や実施例2に示すプライマーペアを用いる。

[0089]

上記のようなプライマーを用いて、HpaIIで処理された遺伝子試料に対してPCRを実施し、遺伝子増幅量確認段階で、例えば電気泳動によりPCR産物の増幅量を確認する。遺伝子試料中に、メチル化されたSHP1遺伝子があれば、HpaIIは切断できないので、PCRにより目的のサイズのPCR産物が検出できる。一方、メチル化されたSHP1遺伝子が無ければ、HpaIIによりDNAが切断されPCR産物は検出できない。【0090】

このように、上記制限酵素確認法を用いれば、メチル化感受性制限酵素を用いて検体試料から得られた遺伝子試料に含まれるSHP1遺伝子の切断を試み、さらにPCRを用いて増幅してから、得られるPCR産物の増幅量を確認することができる。それゆえ、検体試料から微量のSHP1遺伝子さえ得られれば、SHP1遺伝子のメチル化を検出することが可能である。そのため、検体試料中に造血器腫瘍細胞がごく微量しか存在していなくても迅速に高い検出感度で、しかも高特異的に造血器腫瘍細胞を検出することが可能となる 40

[0091]

なお、本実施の形態で説明した上記検出方法には、他の工程(プロセス)や他の段階(ステップ)が含まれていてもよいことは言うまでも無い。例えば、SHP1遺伝子メチル化確認工程において、制限酵素反応やPCR反応を円滑に進めるために、得られた遺伝子試料等を精製する精製段階が含まれていてもよい。

[0092]

本発明には、上述した造血器腫瘍細胞検出方法だけでなく、該検出方法を実施するための 検出キットが含まれる。具体的には、前記SHP1抗体、前記メチル化感受性制限酵素、 前記各プライマー、前記SHP1遺伝子陽性およびメチル化陰性対照DNA等を含む構成 50 を挙げることができる。特に、(1)上記SHP1抗体、および(2)メチル化感受性制限酵素と、PCR用プライマーと、前記SHP1遺伝子陽性およびメチル化陰性対照DNAとの組み合わせに分けた場合には、(1)および(2)の少なくとも一方が含まれていると好ましい。また、SHP1遺伝子産物定量工程とSHP1遺伝子メチル化確認工程の順番はどちらが先であっても良い。

[0093]

さらに、上記検出キットには、必要に応じて、他の各種試薬類が含まれていてもよい。例えば、ヌクレオチドモノマー、ポリメラーゼ、バッファー等のPCR反応用試薬、および、バッファー等の制限酵素反応用試薬の少なくとも一方が含まれていてもよい。

[0094]

より具体的に、各工程または段階ごとに用いられる試薬等について説明する。まず、遺伝子産物定量工程では、蛋白質定量法の場合、酵素抗体法およびウエスタンブロッティング法の何れであっても、SHP1抗体およびその検出試薬が少なくとも用いられる。また、mRNA定量法の場合、RT-PCR法やreal time RT-PCR法を用いる場合、SHP1 c DNA検出用プライマーおよびTaq DNAポリメラーゼ反応試薬が少なくとも用いられる。

[0095]

次に、本実施の形態におけるSHP1遺伝子メチル化確認工程では、メチル化感受性制限酵素によりメチル化を確認するため、まず、遺伝子切断試行段階にて、メチル化感受性制限酵素、メチル化非感受性制限酵素、およびこれらの反応試薬が少なくとも用いられる。次に、遺伝子増幅試行段階では、プライマー、Taa DNAポリメラーゼ反応試薬、システム検討用SHP1遺伝子メチル化陽性DNAが少なくとも用いられる。次に、遺伝子増幅量確認段階では、SHP1遺伝子メチル化陽性およびメチル化陰性対照DNAを用いた反応産物を電気泳動のコントロールとして少なくとも使用することができる。

[0096]

このように、本発明にかかる検出キットでは、前述した造血器腫瘍細胞検出方法を実施するために好ましい薬剤や標本等が含まれている。そのため、上記検出キットを用いることで、本発明にかかる造血器腫瘍細胞検出方法を容易かつ簡素に実施することができ、本発明を臨床検査産業や医薬品産業等の産業レベルで利用することが可能となる。

[0097]

〔実施の形態2〕

本発明における実施の他の形態について図25ないし図47に基づいて説明すれば以下の通りである。なお、本発明はこれに限定されるものではない。また、説明の便宜上、実施の形態1と重複する説明は適宜省略する。

[0098]

前記実施の形態1では、SHP1遺伝子メチル化確認工程に、メチル化感受性制限酵素を用いる制限酵素確認法を用いたが、本発明は、これに限定されるものではなく、本実施の形態では、例えば、遺伝子修飾段階とメチル化シトシン含有判定段階とを含む、重亜硫酸塩を用いてDNAを修飾する方法(以下、説明の便宜上、DNA修飾法と称する)を好適に用いることができる。

[0099]

DNAを重亜硫酸塩(Bisulfite)で処理すると、シトシンはウラシルに変換される。具体的には、図25に示すように、シトシンが重亜硫酸塩によりスルホン化(Sulphonation)され、さらに加水分解により脱アミノ化(Hydrolytic deamination)され、さらに、アルカリ存在下での脱スルホン化(Alkali desulphonation)により、ウラシルに変換される。このウラシルはPCR後、チミンに置き変わる。これに対して、メチル化されたシトシン(5'ーメチルシトシン)は重亜硫酸塩によって変換されない。そこで、本実施の形態では、この重亜硫酸塩処理後の塩基配列の違いを利用して、後述するように、SHP1遺伝子のメチル化の有無を検出する。

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[0100]

次に、本実施の形態におけるSHP1遺伝子メチル化確認工程、すなわちDNA修飾法によるSHP1遺伝子のメチル化の確認について具体的に説明する。

[0101]

まず、遺伝子修飾段階として、造血器細胞を含む前記検体試料から得られた遺伝子試料を重亜硫酸塩で処理する。この段階では、上述したように、メチル化されていないシトシンのみがウラシルに変換されるので、例えば、それゆえ、DNAを重亜硫酸塩処理すると、図26に示すように、メチル化された(図中円で囲んだMで示す)シトシンはシトシンのままで残存するが、メチル化されていないシトシンはウラシル(U)に変換される。

[0102]

上記遺伝子修飾段階で用いられる重亜硫酸塩としては、特に限定されるものではないが、例えば、重亜硫酸ナトリウム(Na2S2O5、メタ重亜硫酸ナトリウム、二亜硫酸ナトリウムまたはピロ亜硫酸ナトリウムともいう)を好適に用いることができる。さらに、重亜硫酸化合物とともに尿素が併用されてもよい。

[0103]

次に、メチル化シトシン含有判定段階として、重亜硫酸塩で処理された遺伝子試料に含まれる、SHP1遺伝子の塩基配列中のシトシンの有無を判定する。重亜硫酸塩処理物中のSHP1遺伝子にシトシンが含まれているということは、処理前のSHP1遺伝子には、メチル化されたシトシンが含まれていることになる。それゆえ、シトシンが存在すれば、元の検体試料中に造血器腫瘍細胞が含まれていることになる。

[0104]

上記メチル化シトシン含有判定段階で実施される、SHP1遺伝子の塩基配列中のシトシンの有無を判定する方法としては特に限定されるものではないが、具体的には、1)メチル化シトシンをPCRにより検出する方法、2)メチル化シトシンを遺伝子の塩基配列の決定により検出する方法、または、3)メチル化シトシンを含む塩基配列を識別する方法のうち、少なくとも何れかの手法を好ましく用いることができる。

[0105]

より具体的には、まず、1) メチル化シトシンをPCRにより検出する方法としては、メチル化特異的PCR (Methylation Specific PCR) を挙げることができる。

[0106]

上記メチル化特異的PCR法は、メチル化されたDNAに特異的でかつCG配列を含む塩基配列をプライマーとして設定する。メチル化されたシトシンが存在していればPCRにより増幅が可能となり、それゆえメチル化されたSHP1遺伝子を検出することができる

[0107]

上記メチル化特異的 P C R 法は、従来公知の方法(例えば、P r o c . N a t l . A c a d . S c i . U S A 9 3 , 9 8 2 1 - 9 8 2 6 , (1 9 9 6) 等の文献に開示されている方法)を好適に用いることができ、その具体的な工程や試薬類、条件等は特に限定されるものではない。なお、D N A の精製過程ではエタノール沈澱法やG l a 40 s s b e a d s 法を用いた方法等を用いることができ、また、蛍光ラベルしたプライマーを用いれば、P C R の検出を容易にすることができる。

[0108]

次に、2)遺伝子の塩基配列の決定によりメチル化シトシンを検出する方法、すなわちSHP1遺伝子のシークエンシングでは、CG配列を含まない領域にプライマーを設定しPCRを実施する。得られるPCR産物の中には、メチル化をされているもの(CG配列のままで存在)とメチル化されていないもの(TG配列に変換されている)が含まれている可能性がある。これをシークエンシングすることにより、CG配列すなわちメチル化の存在を検討する。

[0109]

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上記SHP1遺伝子のシークエンシングも、従来公知の方法(例えば、Proc. Nat1. Acad. Sci. USA 89, 1827-1831 (1992)等の文献に開示されている方法)を好適に用いることができ、その具体的な工程や試薬類、条件等は特に限定されるものではない。なお、上記プライマーとしては、メチル化されたDNAに特異的な配列(CG配列を含む)を有するプライマーを用いることも可能である。

[0110]

この方法もPCRを用いているので、検体試料から微量のSHP1遺伝子さえ得られれば、SHP1遺伝子のメチル化を検出することが可能である。そのため、検体試料中に造血器腫瘍細胞がごく微量しか存在していなくても高い検出感度で高特異的に造血器腫瘍細胞 10を検出することが可能となる。また、シークエンシングを利用することにより具体的な配列を決定するので、メチル化の程度をより明確化することも可能となる。

[0111]

次に、3)シトシンを含む塩基配列を識別する方法としては、Ms-SnuPE法、重亜硫酸塩SSCP法、メチルライト法、蛍光溶解曲線分析法、COBRA法等を挙げることができる。

[0112]

上記Ms-SnuPE(Methylation-sensitive Single Nucleotide Primer Extension)法は、メチル化されたDN Aに特異的なプライマーを用いてPCRを実施する方法である。ただし、プライマーに挟 20まれた領域でのメチル化の有無が判らないので、検出したいCG配列に隣接するポリヌクレオチドを作成しPCR産物とアニールさせる。放射性同位元素の存在下でDNAを合成した時に、32P-dCTPを取り込めば、そこはCG配列であるためメチル化されているシトシンが存在することになる。一方、DNAを合成した時に、32P-dTTPを取り込めば、そこはTG配列であるためメチル化はされていなかったことになる。

[0113]

上記Ms-SnuPE法は、従来公知の方法(例えば、Nucleic Acids Research 25, 2529-2531, (1997) 等の文献に開示されている方法)を好適に用いることができ、その具体的な工程や試薬類、条件等は特に限定されるものではない。

[0114]

上記重亜硫酸塩SSCP(Bisulfite-SSCP)法も、メチル化されたDNAに特異的なプライマーを用いてPCRを実施する方法であるが、プライマーに挟まれた領域でのメチル化の有無が判らない。そこで、PCR産物を1本鎖DNAに変性後、SSCP(Single Strand Conformational Polymorphism)法を用いて電気泳動し、1本鎖DNAの移動度の違いから、SHP1遺伝子のメチル化の程度を判定する。

[0115]

[0116]

他に、メチルライト(Methyl-light)法や、蛍光溶解曲線分析(FluorescenceMelting Curve Analysis)法等も挙げられる。これら方法も、何れもメチル化されたDNAに特異的なプライマーを用いてPCRを実施する方法であるが、プライマーに挟まれた領域でのメチル化の有無が判らない。そこで、内側の調べたい領域について、メチル化特異的なポリヌクレオチドを作成し、このメチル化特異的ポリヌクレオチドが1本鎖にしたPCR産物とどの程度アニール(2本鎖重合)反応するかを検討することにより、上記PCR産物中のメチル化の量を判定する。

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[0117]

上記メチルライト法は、具体的には、例えば、Nucleic Acids Research 28(8), E32, (2000) 等の文献に開示されている方法を、上記蛍光溶解曲線分析は、具体的には、例えば、Clinical Chemistry 47, 1183-1189, (2001) 等の文献に開示されている方法を好適に用いることができる。

[0118]

上述した各方法は、PCRを用いているので、検体試料から微量のSHP1遺伝子さえ得られれば、SHP1遺伝子のメチル化を検出することが可能である。そのため、検体試料中に造血器腫瘍細胞がごく微量しか存在していなくても高い検出感度で高特異的に造血器 10腫瘍細胞を検出することが可能となる。

[0119]

上記 C O B R A 法 (C o m b i n e d B i s u l f i t e R e s t r i c t i o n A n a l y s i s 、あるいは、B i s u l f i t e P C R f o l l o w e d b y r e s t r i c t i o n a n a l y s i s 等とも称される) では、例えば、C G C G 配列がメチル化を受けていると、重亜硫酸処理後もC G C G 配列のままで残存するが、メチル化されていないとT G T G 配列に変換される。そこで、上記 C G C G 配列のみを切断する制限酵素等を利用することで、電気泳動ゲル上のバンドパターンを解析して、S H P 1 遺伝子のメチル化の有無を判定および定量化することができる。

[0120]

上記 C O B R A 法も、従来公知の方法(例えば、N u c l e i c A c i d s R e s e a r c h 2 5 , 2 5 3 2 - 2 5 3 4 , (1 9 9 7) 等の文献に開示されている方法)を好適に用いることができ、その具体的な工程や試薬類、条件等は特に限定されるものではない。勿論、この方法でもP C R が用いられるので、上述したP C R による利点が得られるだけでなく、制限酵素処理と電気泳動とを用いるので、バンドパターンの解析さえ明確化しておれば、容易にS H P 1 遺伝子のメチル化を確認することができるという利点もある。

[0121]

このように、本実施の形態におけるDNA修飾法では、メチル化シトシン含有判定段階でPCRを用いているが、このPCRで用いるプライマーの設計方法について以下に説明す 30 る。

[0122]

上述したように、DNAを重亜硫酸塩処理するとシトシンはウラシルに変換されるが、メチル化されたシトシンは変換されずに保存される。ここで、細胞内でメチル化を受ける可能性のあるシトシンは、5′配列側からCGと並ぶCG配列(5′-CG-3′)のシトシン(C)のみである。そのため、重亜硫酸塩処理により、上記CG配列以外のシトシンは全てチミン(T)に変換されてしまう。そこで、全てのCG配列がメチル化を受けたものとしてSHP1遺伝子の塩基配列を変換し、プライマーを設定する。なお、DNA中のウラシルはチミンとして認識され、PCRによりチミンに置換されることになる。

[0123]

まず、プライマーを計画するDNA鎖に関する条件を設定する。SHP1遺伝子の塩基配列において、センス鎖またはアンチセンス鎖の何れも、上記CG配列のみがメチル化を受けたとして、その他の塩基配列におけるシトシンが全てチミンに変換された配列を想定する。

[0124]

具体的には、図27~図36および配列番号5に示す塩基配列が、図1~図10および配列番号1に示すSHP1遺伝子のゲノムDNA(ワイルドタイプ)のセンス鎖に対応する、重亜硫酸塩処理後の塩基配列(以下、説明の便宜上、センス鎖変換配列とする)であり、図37~図46および配列番号6に示す塩基配列が、図11~図20および配列番号2に示すSHP1遺伝子のゲノムDNA(ワイルドタイプ)のアンチセンス鎖とするに対応50

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する、重亜硫酸塩処理後の塩基配列(以下、説明の便宜上、アンチセンス鎖変換配列とする)である。これらセンス鎖変換配列とアンチセンス鎖変換配列とは、重亜硫酸塩処理により相補的ではなくなる。

[0125]

なお、図27~図36および配列番号5、並びに、図37~図46および配列番号6の塩基配列は、CG配列が100%メチル化されていると想定した場合に、重亜硫酸塩処理を受けたものとしての塩基配列であり、実際には細胞中で100%のメチル化が生じるとは考えられないため、本発明において検出し得る可能性としての塩基配列として例示する。

そして、(I)上記センス鎖変換配列に対して、フォワードプライマー(FWプライマー 10)およびリバースプライマー(RVプライマー)を作成するか、あるいは、(II)上記アンチセンス鎖変換配列に対して、FWプライマーおよびRVプライマーを作成する。この場合、同じ場所でもプライマー配列はそれぞれ異なる。

[0 1 2 7]

[0126]

次に、プライマーを計画する領域に関する条件を設定する。(i)メチル化されたDNAのみを直接PCRで増幅するために、CG配列を含む塩基配列に対してプライマーを作成するか、(ii)メチル化されたもの、されていないものを区別なくPCRで増幅するために、CG領域を含まない配列に対してプライマーを作成する。なお、(ii)の場合は、後でシークエンシングかその他の方法を実施し、メチル化を判定する。

[0128]

したがって、DNA修飾法で用いられるプライマーの設計には、上記DNA鎖に関する条件(I)および条件(II)と、領域に関する条件(i)および条件(ii)とを掛け合わせた4通りの設計方法がある。

[0129]

ここで、(i)の場合、プライマーの場所が都合良くメチル化を受けていれば検出されるが、その場所ではなく近隣領域のみメチル化を受けているような場合には、メチル化が存在するのにも関わらず検出不可能となる。そこで、(ii)のように、メチル化の有無に関わらずPCRで増幅後、各プライマーに囲まれた領域内のメチル化、すなわちCG配列の有無を検定することで、確実にSHP1遺伝子のメチル化を検出することができる。そのため、本実施の形態におけるSHP1遺伝子のメチル化の判定には、検出用のプライマ 30 一の場所のみならず、遺伝子配列の情報そのものが重要となる。

[0130]

また、CG配列がメチル化されていないと、重亜硫酸塩処理によりTG配列に変換されるが、このTG配列を含む塩基配列に対して作成されるプライマー(Unmethylated primer)は、メチル化を受けていないDNAの存在を証明するコントロールとして用いることができる。また、重亜硫酸塩処理が不十分な場合には、シトシンがウラシルに変換されていないワイルドタイプのSHP1遺伝子が混入することになる。そこで、重亜硫酸塩処理が十分完全になされたか否かのコントロールとして、ワイルドタイプの塩基配列を有するプライマー(Wild type primer)を用いることができる。

[0131]

なお、上述したメチル化シトシン含有判定段階では、PCRにより増幅された遺伝子の確認に、前記実施の形態1における遺伝子増幅量確認段階と同様の方法、例えば、電気泳動法を用いてマーカーと比較することにより遺伝子の増幅量を確認したり、さらに電気泳動後に得られたDNAバンドをメンブレンにプロッティングしたりする手法が挙げられる。 勿論、これら手法に限定されるものではなく、また、上記電気泳動法やブロッティングの方法についても従来公知の手法を好適に用いることができ、特に限定されるものではない

[0132]

換言すれば、本実施の形態におけるDNA修飾法によるSHP1遺伝子メチル化確認工程 50

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でも、前記実施の形態1における制限酵素確認法による場合と同様、遺伝子増幅量確認段階が含まれていても良い。

[0133]

次に、本実施の形態にかかる検出方法の好ましい一例について、より具体的に説明する。

まず、SHP1遺伝子産物定量工程により、前述した手法を用いて検体試料中に含まれるSHP1蛋白質およびSHP1mRNAの少なくとも一方を定量する。このプロセスで定量されたSHP1蛋白質が、標準よりも大幅に減少していたり、ほとんどSHP1遺伝子産物が発現していなかったりした場合には、検体試料中に造血器腫瘍細胞が含まれている可能性が高くなる。

[0135]

次に、SHP1遺伝子メチル化確認工程で、前記DNA修飾法により、検体試料から調製した遺伝子試料中のSHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認する。具体的には、遺伝子修飾段階にて、例えば重亜硫酸ナトリウムを用いて、上記検体試料から得られた遺伝子試料をで処理する。

[0136]

次に、遺伝子増幅試行段階に移行するが、このステップでは、前述したプライマーの設計方法に基づいて、PCR用のプライマーを設定する。

[0137]

具体的には、メチル化特異的PCRでは、図47(a)に示すように、例えば、23塩基 20 対のワイルドタイプDNA(図中上がセンス鎖で下がアンチセンス鎖)を想定し、ワイルドタイプDNAのCG配列に100%メチル化があるとする。この場合、重亜硫酸塩処理すると、図47(b)に示すように、センス鎖とアンチセンス鎖は相補的ではなくなる。そこで、図47(c)または(d)に示すように、センス鎖またはアンチセンス鎖に対してFWプライマーおよびRVプライマーを作成する。

[0138]

なお、上記メチル化特定 P C R においては、 P C R 用プライマーとして、 具体的には、 例えば、 後述する 実施例 4 や実施例 5 に示すプライマーペアを用いる。 上記のようなプライマーを用いて、 重亜硫酸ナトリウムで処理された遺伝子試料に対してメチル化特異的 P C R を実施し、 例えば電気泳動により P C R 産物の増幅量を確認する。

[0139]

このように、上記DNA修飾法を用いれば、重亜硫酸塩を用いて検体試料から得られた遺伝子試料を処理すると、塩基配列中のシトシンはウラシルに変換されるが、メチル化されたシトシンは変換されない。そのため、遺伝子修飾段階後のSHP1遺伝子の塩基配列中にシトシンが含まれるか否かを判定するのみで、SHP1遺伝子のメチル化を検出することができる。そのため、単純なメカニズムで迅速かつ高特異的に造血器腫瘍細胞を検出することが可能となる。

[0140]

次に、SHP1遺伝子産物定量工程により、前述した手法を用いて検体試料中に含まれるSHP1蛋白質およびSHP1mRNAの少なくとも一方を定量する。このプロセスで定 40量されたSHP1遺伝子産物が、標準よりも大幅に減少していたり、ほとんど発現していなかったりした場合には、検体試料中に造血器腫瘍細胞が含まれている可能性が高くなる

[0141]

なお、本実施の形態で説明した上記検出方法には、前記実施の形態 1 の検出方法と同様に、他の工程(プロセス)や他の段階(ステップ)が含まれていてもよいことは言うまでも無い。

[0142]

本発明には、上述した造血器腫瘍細胞検出方法だけでなく、該検出方法を実施するための 検出キットが含まれる。具体的には、遺伝子処理レベルまで精製された重亜硫酸塩と前記 50 プライマー、および前記SHP1抗体を含む構成を挙げることができる。つまり、本発明にかかる検出キットでは、上記重亜硫酸塩、プライマー、およびSHP1抗体を、(1)上記SHP1抗体、(2)重亜硫酸塩と、該重亜硫酸塩で処理された遺伝子試料に含まれるSHP1遺伝子の塩基配列中のシトシンの有無の判定用プライマー、および(3)配列番号3に示すSHP1遺伝子cDNAの塩基配列の全長またはその一部を検出するPCR用のプライマーに分けた場合、(1)、(2)および(3)のうち、少なくとも何れか一つを含むことが好ましい。

[0143]

さらに、上記検出キットには、配列番号3に示すSHP1遺伝子cDNAの塩基配列の全長またはその一部と相同性を持つノーザンブロッティング用プローブ、または、シトシン 10を含む塩基配列を認識する制限酵素およびSHP1遺伝子のメチル化陽性及びメチル化陰性対照DNAを用いた電気泳動用マーカーが含まれていてもよく、さらには、ヌクレオチドモノマー、ポリメラーゼ、バッファー等のPCR反応用試薬、および、バッファー等の制限酵素反応用試薬の少なくとも一方が含まれていてもよい。

[0144]

より具体的に、各工程または段階ごとに用いられる試薬等について説明する。まず、遺伝子産物定量工程では、前記実施の形態1で例に挙げたものと同様であるのでその説明は省略する。

[0145]

次に、本実施の形態における S H P 1 遺伝子メチル化確認工程では、重亜硫酸塩処理によ 20 りメチル化を確認するため、まず、遺伝子修飾段階にて、各種重亜硫酸塩等の試薬が少なくとも用いられる。次に、メチル化シトシン含有判定段階では、メチル化シトシンを P C R により検出する方法を用いる場合には、メチル化配列特異的プライマー、およびTaaDNAポリメラーゼ反応試薬が少なくとも用いられる。また、遺伝子の塩基配列の決定によりメチル化シトシンを検出する方法、あるいはシトシンを含む塩基配列を認識する方法では、各具体的な方法に応じて公知の試薬類を用いる。

[0146]

このように、本実施の形態にかかる検出キットでも、前記実施の形態1の検出キットと同様、前述した造血器腫瘍細胞検出方法を実施するために好ましい薬剤や標本等が含まれている。そのため、上記検出キットを用いることで、本発明にかかる造血器腫瘍細胞検出方 30 法を容易かつ簡素に実施することができ、本発明を臨床検査産業や医薬品産業等の産業レベルで利用することが可能となる。

[0147]

なお、本発明は、上述した各実施の形態に限定されるものではなく、請求項に示した範囲で種々の変更が可能であり、異なる実施の形態にそれぞれ開示された技術的手段を適宜組み合わせて得られる実施の形態についても、本発明の技術的範囲に含まれることはいうまでもない。

[0148]

【実施例】

以下、図48ないし図52に基づいて、本発明の具体的な実施例について説明する。なお 40、本発明はこれに限定されるものではない。

[0149]

〔実施例1〕

ナチュラルキラー細胞リンパ腫を含む検体試料を用い、Towbin H. et al: Proc. Natl. Acad. Sci. USA76, 4350, (1979) に開示されている方法にしたがってウエスタンプロッティングを実施した。なお、SHP1抗体として#SH-PTP1 (D-11): sc7289 (Santa Cruz Biotechnology Inc. 製)を用いた(SHP1遺伝子産物定量工程・蛋白質定量法)。

[0150]

その後、SHP1遺伝子メチル化確認工程に移行した。まず、メチル化感受性制限酵素としてHpaIIを用いて、上記検体試料から調製した遺伝子試料を37℃4時間で処理した(遺伝子切断試行段階)。

[0151]

次に、HpaIIで処理した遺伝子試料をPCRで増幅した(遺伝子増幅試行段階)。このとき用いたプラーマーペアは、配列番号 7 および図 4 8 (a) に示す 1 9 塩基のプライマーREP-S1 と、配列番号 8 および図 4 8 (b) に示す 2 0 塩基のプライマーREP-AS1 との組み合わせとした。このプライマーペアを用いた場合、配列番号 9 および図 4 8 (c) に示すように、SHP1遺伝子のセンス鎖の配列(配列番号 1 および図 1 ~図 1 0 参照)における、7 4 4 1 塩基から 7 5 6 6 塩基までの 1 2 6 塩基の塩基配列が検出 10 される。

[0152]

なお、図48(c)におけるカッコ内の「#(番号)」は、上記SHP1遺伝子のセンス鎖における塩基の位置を示しており、下線部はプライマーREP-S1およびREP-AS1の対応位置、並びにHpaIIの認識切断部位の位置を示している。また、プライマーREP-AS1は、上記REP-AS1の下線部の領域におけるアンチセンス鎖の配列に対してデザインされたものである。

[0153]

その後、アガロースゲルで電気泳動してから、得られたDNAバンドをナイロンメンブレンにブロッティングしてSHP1遺伝子の増幅を確認した(遺伝子増幅量確認工程)。 【 0 1 5 4】

次に、Towbin H. et al: Proc. Natl. Acad. Sci. USA76, 4350, (1979) に開示されている方法にしたがってウエスタンブロッティングを実施した。なお、SHP1抗体として#SH-PTP1 (D-11):sc7289 (Santa Cruz Biotechnology Inc. 製)を用いた(SHP1遺伝子産物定量工程・蛋白質定量法)。

[0155]

上記SHP1遺伝子メチル化確認工程とSHP1遺伝子産物定量工程との結果から検体試料中の造血器腫瘍細胞を検出した。

[0156]

〔実施例2〕

遺伝子増幅試行段階で、プライマーペアとして、配列番号10および図49(a)に示す 21塩基のプライマーREP-S2と、配列番号11および図49(b)に示す21塩基 のプライマーREP-AS2との組み合わせを用いた以外は、前記実施例1と同様にして 検体試料中の造血器腫瘍細胞の有無を検出した。

[0157]

上記プライマーペアを用いた場合、配列番号 1 2 および図 4 9 (c) に示すように、SHP 1 遺伝子のセンス鎖の配列(配列番号 1 および図 1 \sim 図 1 0 参照)における、 6 8 5 8 塩基から 7 0 8 4 塩基までの 2 2 7 塩基の塩基配列を検出することができる。

[0158]

なお、図49(c)におけるカッコ内の「#(番号)」も、上記SHP1遺伝子のセンス鎖における塩基の位置を示しており、下線部はプライマーREP-S2およびREP-AS2の対応位置、並びにHpaIIの認識切断部位の位置を示している。また、プライマーREP-AS2は、上記REP-AS2の下線部の領域におけるアンチセンス鎖の配列に対してデザインされたものである。

[0159]

〔 実 施 例 3 〕

RT-PCRによるmRNA定量法を用いてSHP1遺伝子産物定量工程を実施した以外は、前記実施例1と同様にして検体試料中の造血器腫瘍細胞の有無を検討した。

[0160]

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すなわち、前記検体試料から全細胞内のRNAを調製してから逆転写酵素により逆転写した。その後、SHP1特異的プライマーペアを用いてPCRにより増幅した。上記SHP1特異的プライマーペアとしては、配列番号13および図50(a)に示す23塩基のプライマーSHP-PR1と、配列番号14および図50(b)に示す25塩基のプライマーSHP-PR1との組み合わせを用いた。

[0161]

〔実施例4〕

real time RT-PCRによるmRNA定量法を用いてSHP1遺伝子産物定量工程を実施した以外は、前記実施例3(すなわち前記実施例1)と同様にして検体試料中の造血器腫瘍細胞の有無を検討した。上記SHP1特異的プライマーペアとしては、配 10列番号15および図51(a)に示す20塩基のプライマーSHP-LF1と、配列番号16および図51(b)に示す20塩基のプライマーSHP-LR1を用いた。

[0162]

〔 実 施 例 5 〕

Proc.Natl.Acad.Sci.USA 93, 9821-9826, (1996)に開示されている方法にしたがってメチル化特異的PCRを用いてSHP1遺伝子メチル化確認工程を実施した以外は、前記実施例1と同様にして検体試料中の造血器腫瘍細胞の有無を検討した。なお、重亜硫酸塩としては、重亜硫酸ナトリウムを用いた。

[0163]

また、上記メチル化特異的PCRにおけるプライマーペアとしては、配列番号17および図52(a)に示す24塩基のプライマーMF2と、配列番号18および図52(b)に示す21塩基のプライマーMR2との組み合わせを用いることができる。このプライマーペアを用いた場合、配列番号19および図52(c)に示すように、SHP1遺伝子のセンス鎖の配列(配列番号1および図1~図10参照)における、7037塩基から7195塩基までの159塩基の塩基配列を検出することができる。

[0164]

なお、図52(c)におけるカッコ内の「#(番号)」は、上記SHP1遺伝子のセンス鎖における塩基の位置を示しており、下線部はプライマーMF2およびMR2の対応位置を示している。ただし、上記各プライマーはメチル化されているDNAのみを検出できる 30ように設計されているので、その塩基配列は、上記下線部の塩基配列とは少し異なっている。また、プライマーMR2は、上記MR2の下線部の領域におけるアンチセンス鎖の配列に対してデザインされたものである。

[0165]

〔実施例6〕

検体試料として、診断用の骨髄(BM)検体と、ALL(急性リンパ芽球性白血病)患者の末梢血(PB)検体とを用いた。ALL患者から得られたBM検体は少なくとも70%の比で芽細胞を含んでいた。また、これら検体試料に対する対照試料は、化学療法によって達成された血液学的完全寛解の後に得られた。

[0166]

上記検体試料を用いてマイクロサテライト解析を行った。このときのPCR反応では、5 '側のプライマーを、5 'ーi o d o a c a t a m i d e f l u o r e s c e i n でラベルし、反応系は、1 0 p m o l のそれぞれのプライマー、4 0 n g のゲノムDNA、1 × P C R バッファー、2 0 0 μ M のそれぞれの d N T P と、0 . 5 u n i t の T a q D N A p o l y m e r a s e を含む 2 0 μ l の系とした。得られた P C R 産物は、A B I P r i s m 3 1 0 0 D N A s e q u e n c e r (A p p l i e d B i o s y s t e m s , F o s t e r C i t y , C A) にかけ、G e n e s c a n A n a l y s

is software ver 3.7 (Applied Biosystems) で

解析を行った。 【0167】

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その結果、図53(a)・(b)に示すように、D12S336マーカーおよびD12S356マーカーによってSHP1遺伝子のLOHの有無を確認できることがわかった。本実施例の結果では、これらマーカーのうちテロメア側のD12S356マーカーでは、有意な結果が得られた19症例中15例(79%)にLOHが認められた。また、セントロメア側のD12S36でーカーでは、16症例中6例(38%)にLOHが認められた

[0168]

上記何れの実施例の結果も、検体試料から十分に造血器腫瘍細胞を検出することができた。それゆえ、本発明は、複数の診断手法を併用しなくても造血器腫瘍細胞を容易かつ迅速に検出することができることがわかった。

[0169]

【発明の効果】

以上のように、本発明にかかる造血器腫瘍細胞検出方法は、造血器細胞を含む検体試料中に含まれる、造血器細胞に特異的なSHP1蛋白質およびそのmRNAの少なくとも一方を定量するSHP1遺伝子産物定量工程と、上記検体試料から得られる、上記SHP1蛋白質をコードするSHP1遺伝子の塩基配列中に含まれるCpG島のメチル化を確認するSHP1遺伝子メチル化確認工程と、上記検体試料に含まれるSHP1遺伝子の異型接合性喪失(LOH)の有無を確認するSHP1遺伝子LOH確認工程とを含む方法である。

[0170]

[0171]

本発明の方法または構成によれば、SHP1遺伝子の発現を、遺伝子DNAの修飾とmRNAと蛋白質と対立遺伝子の喪失という最大で四重のマーカーを用いて判定できることになる。すなわち、SHP1遺伝子の発現低下という一つの造血器腫瘍細胞特異的な現象を4段階で確認することができるため、非常に高い特異性で造血器腫瘍細胞を検出することができる。よって、本発明を用いることで、造血器細胞を含む微量の検体試料から造血器腫瘍細胞を容易かつ迅速に検出することができる。

[0172]

それゆえ、本発明における悪性リンパ腫・白血病の高感度検出法を用いると、一般集団検診による造血器腫瘍の早期発見、診断および治療後のモニタリングや再発の早期発見が可能になり、これら疾患を発症した家族等血縁者における発症危険度の予測等に本発明を利用することも可能となる。その結果、本発明を臨床検査産業や医薬品産業等の産業レベルで利用することが可能となるという効果を奏する。

[0173]

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<213> Artificial Sequence

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<223> Description of Artificial Sequ Synthesized Primer Sequence

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<210>9

<211> 126

<212> DNA

<213> Homo sapiens

<400>9

cagging cagting cage congains grant acting grant category control of the category category can be caused as a constant of the cagging can be caused as a care can be care can be caused as a care can be caused as a care can be caused as a care can be care can be care can be care can be care can

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 $\langle 2 1 0 \rangle 1 0$

<211>21

<212> DNA

<213> Artificial Sequence

(220)

<223> Description of Artificial Sequ Synthesized Primer Sequence

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10

<210> 11

<211>21

<212> DNA

<213> Artificial Sequence

<220>

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<223> Description of Artificial Sequ Synthesized Primer Sequence

<400>11

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<210>12

 $\langle 211 \rangle 227$

<212> DNA

<213> Homo sapiens

<400>12

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<210>13

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequ Synthesized Primer Sequence

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<210>14

<211>25

<212> DNA

<213> Artificial Sequence

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(210) 15
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<211> 20

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequ Synthesized Primer Sequence

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<400>15

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<210>16

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<213> Artificial Sequence

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<223> Description of Artificial Sequ Synthesized Primer Sequence

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(211) 24

<212> DNA

<213> Artificial Sequence

< 220>

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 $\langle 210 \rangle 18$

 $\langle 211 \rangle 21$

<212> DNA

<213> Artificial Sequence

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<220>

<223> Description of Artificial Sequ Synthesized Primer Sequence

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30

<210>19

 $\langle 211 \rangle 159$

 $\langle 212 \rangle$ DNA

<213> Homo sapiens

40

< 4 0 0 > 1 9

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【図面の簡単な説明】

【図1】本発明にかかる造血器腫瘍細胞検出方法で用いられるSHP1遺伝子ゲノムDNAのセンス鎖の塩基配列を示す塩基配列図である。

【図2】図1に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の続きを示す配列図である。

【図3】図1・図2に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の 続きを示す配列図である。

【図4】図1~図3に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の続きを示す配列図である。

【図 5 】図 1 ~図 4 に示す S H P 1 遺伝子のゲノム D N A におけるセンス鎖の塩基配列の続きを示す配列図である。

【図 6 】 図 1 ~ 図 5 に示す S H P 1 遺伝子のゲノム D N A におけるセンス鎖の塩基配列の続きを示す配列図である。

【図7】図1~図6に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の 続きを示す配列図である。

【図8】図1~図7に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の 続きを示す配列図である。

【図9】図1~図8に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の 続きを示す配列図である。

【図10】図1~図9に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖の塩基配列の続きを示す配列図である。

【図11】本発明にかかる造血器腫瘍細胞検出方法で用いられるSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列を示す塩基配列図である。

【図12】図11に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図13】図11・図12に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図14】図11~図13に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図15】図11~図14に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図16】図11~図15に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図17】図11~図16に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図18】図11~図17に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図19】図11~図18に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図20】図11~図19に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖の塩基配列の続きを示す配列図である。

【図21】本発明にかかる造血器腫瘍細胞検出方法で用いられるSHP1遺伝子のcDNAの塩基配列を示す塩基配列図である。

【図22】本発明にかかる造血器腫瘍細胞検出方法で用いられるSHP1蛋白質の概略構造を示す模式図である。

【図23】図22に示すSHP1蛋白質のアミノ酸配列を示すアミノ酸配列図である。

【図24】図1に示すSHP1遺伝子のゲノムDNA(センス鎖)において、CpG島でメチル化されるCG配列の部位を示す塩基配列図である。

【図 2 5 】本発明にかかる造血器腫瘍細胞検出方法で用いられる重亜硫酸処理にて、シトシンがウラシルに変換される過程を示す化学反応説明図である。

【図26】本発明にかかる造血器腫瘍細胞検出方法で用いられる重亜硫酸処理により、シトシンがウラシルへ変換され、メチル化されたシトシンが変換されない状態を示す模式図である。

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【図27】本発明にかかる造血器腫瘍細胞検出方法で用いられるSHP1遺伝子ゲノムDNAのセンス鎖に対して、重亜硫酸塩処理した後の塩基配列を示す塩基配列図である。

【図28】図27に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図 2 9 】図 2 7 ・ 2 8 に示す S H P 1 遺伝子のゲノム D N A におけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図30】図27~図29に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図31】図27~図30に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図32】図27~図31に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図33】図27~図32に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図34】図27~図33に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図35】図27~図34に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図36】図27~図35に示すSHP1遺伝子のゲノムDNAにおけるセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図37】本発明にかかる造血器腫瘍細胞検出方法で用いられるSHP1遺伝子ゲノムDNAのアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列を示す塩基配列図である。

【図38】図37に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図39】図37・図38に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図40】図37~図39に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図41】図37~図40に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖 30に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図42】図37~図41に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図43】図37~図42に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図44】図37~図43に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図45】図37~図44に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図46】図37~図45に示すSHP1遺伝子のゲノムDNAにおけるアンチセンス鎖 40に対して、重亜硫酸塩処理した後の塩基配列の続きを示す配列図である。

【図47】(a)~(d)は、それぞれ本発明で用いられるメチル化特異的PCRのステップを示す模式図である。

【図48】(a)・(b)は、本発明の実施の一例である実施例1において用いられるPCR用プライマーを示す塩基配列図であり、(c)は、(a)・(b)で用いられるPCR用プライマーが認識するSHP1遺伝子(ゲノムDNA・センス鎖)の塩基配列を示す塩基配列図である。

【図49】(a)・(b)は、本発明の実施の一例である実施例2において用いられるPCR用プライマーを示す塩基配列図であり、(c)は、(a)・(b)で用いられるPCR用プライマーが認識するSHP1遺伝子(ゲノムDNA・センス鎖)の塩基配列を示す

塩基配列図である。

【図 5 0】 (a)・(b) は、本発明の実施の一例である実施例 3 において用いられる RT-PCR用プライマーを示す塩基配列図である。

【図51】(a)・(b)は、本発明の実施の一例である実施例4において用いられるreal time RT-PCR用プライマーを示す塩基配列図である。

【図52】(a)・(b)は、本発明の実施の一例である実施例5において用いられるメチル化特異的PCR用プライマーを示す塩基配列図であり、(c)は、(a)・(b)で用いられるメチル化特異的PCR用プライマーが認識するSHP1遺伝子(ゲノムDNA・センス鎖)の塩基配列を示す塩基配列図である。

【図53】(a)は、蛍光in situ ハイブリダイゼーション(FISH)を示す 12 図であり、(b)は、ALL患者におけるSHP1遺伝子の異型接合性喪失の解析結果の一つの典型的なデータを示す図である。

【図1】

contotagit gigocoegig teguconant gionicatos unaccacacg gracagaggò otgoatgost cotstitues assossotus coagcounts suggascent testgottas actsocacco octocarate tottagroso gotestoott argagagge tigacottoo sotcoctott geagatgtee ttaagtttge tegettggte asgtootsog sagoocaggs tootgagate goongootst cargonagot ganggogots titotgoogo costsacoot gooscoccat aggeotycty otgytygoag cytygocyce teotyagayt tygoodtocc ttgtgccapt gcceggggg gasaggcott gatgttccag scantastas atgcgootgt santtagnet teststesst etettspass cotsanage constitute ettocotsst tecetotgee titecaggee contococot gancagetee tocotatggt cotggetggg cotsaccets occossisce tascectace tenggeteet eccettooco oggazoagat tgagaggotg gagtgggtoc otcagogoco tgggtgggtg ggootgosoa gggggtacot. cottototge gguactgego tgttaggget tttoottagg cootttggtt tccgcctscg gagaggtito occontiggt igototicot cagooagegt teoticoteg toigticoco tenconstan congruente tetraguite agutocaset geagatocas etescionio ototocoggy ggnaggoggo cotggaccag caggogggos tgctgtacto cogotitiggg gotgongaga agotggoogo tgtgggoggt otogggoong coccgoocca cotgtocttt tootggagac tattagtoca gggtttgtoc otgcagtgcc ATTGGCCTDG CASSCASGAT COASGASSAA STORCTSATT ACTSACCSOT TCTTCCTCAC CTSGCTTGGS CCACTETGCA CARCTRIFICO BOTGGOTCAB COCCOCCCC TECGGCCCTC COCCUTBOCT TCCCCCTCCC TACAGAGAGA TGOTETOCOS TGOgteseto cogregososa toggegtoco agtotoctet tagtitigga gggagggagg gottigitga tgotosotoo gaogigigig asogigagig ogstotgoog otgocotgog cotgittoog glocotetge sottoccott coogceaggt gigaggacoo coggotomot catgotocto igococcitoi timecatiti cocciggaca agigigiato igitototoo atigostito isoticosgo ototgegoto etgottetgo otcotgotta ggacotgtos ocotgggtag otcacascas otcasacata gcagtcagag spendence assengator caustocase pasettotos scauttocoa acatoasset ttagtoccat éttettigit tootticact tesetticce etgeateatt catteaseag gtecetette agostotett atgosoosge tgotettias gatgotegta atactegast ganodagnos gaostegtot otgototoso ggagottaos ttoosgtegg sgettacagu coguacanet asconnetas attegatost tecagattot caganetatt soccagasas tagacagoot tagacagata tagtastica cacctataat cocagosota tagagagata aggogagage attgottgag cocaggaget tgagacongo otggocasta tagtgagac otgiototac assassinag esattagotg guigigging cacacgioot giggitocag

【図2】

120

300

360

420

600

660

720

840

900

860

1020

1080

1140

1200

1280

1320

1380

1440

1500

1680

1740

1800

1980

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【図11】

totocotgae esgectosta agitoesgga ggioggggga etgigasige etgigaegee exempototy tangentity organizate cangoocego ototycgott tygganotyt sotatotoss cotttooses agtaseosco agoacttoot saguestrac tocoscosso titactocco aggacaccag tgittossat seagasgasa coccactcot gacatgggg unguagagy gyacanassa canagonoto ttomogtomy competetto senototyco configurate aggreeatous officerates fulfilled and factorist cooleast of 360 ctgaggagco tgtocotgtt cocgosagag gyracgcago cgggotggco toaggtotgo gottooggot gitamoteot ottottagit cagatotgga actoagoato aggagagigi gatuagggco cocotgaggg tggcocoago tgcaactoca gggtaaggag tgtgggotot aggatoagao agootagatt ogaatooong ootoaotatt contagooot atgaoottag gosagtgeco eagtisotta gtittiotgt cootcagtit cogcosoggi sisatgagas tgapagesgt gootsgactg sectasogtg tgtagtottt tegtgootgg goacatggos 720 agogtetget gagtosotet gooogtotta togtoagoog tostotoggt gottttosoa acceccotgt tatgatoguo otocattita asganggaga otggggagut toacceacgg tomocotoco mogigogagg oggagicocg mocomogico giotgacgga aggoggagot ttcsogogas gozogosogg aggososcas agttsoccat octgocotgo cototggoog occaptosot gggcaagosa agggotoaga ggogsososo ASTEATCCCA GGGCTTTATT 1020 TACAAGAGA BAAGGETTOG COCTGCCTGG GGCCTGGCTG GGCTATATAC AGGCTCAGGG ADABOTOGGO GOGATGCAGC CATITANATT ACAAAAGAAT GOGGCACTCC TAROTTCAGG 1140 THETBACTOT STOCATOOCS AAATOCTTCC ACAGGSTCAG SOCTGAGGST goodstgags 1200 PERACTERE MARTERERE STANCTERS COLUMNIES PROCESSASE STOTEFFECE 1260 gotgerango goagasteng gotgogroca coccatoono coagregang cogteraguo .1320 HERECAGER EXCHERECER EXAMENCED CHERCHERER SECTETACIA TOSCCACCTO AGGACAGGAO COCTCACTTC CTOTTGAGGG AACCCTTGOT CTTCTCCTTG TCTGCTGACC 1440 OCTOCITCIT CACTITOTCO TOCCIOTTET TOTTABIETE CASSITOTCA TACACATCOL 1500 CCTIGIGIOT gengeogest scassesses antheases asttestsea setenzana 1560 CARCADARUE READCORNOA STREORNICO COLOTRODA COCCUETOTOS DARRIESCAD 1620 ageosogato cotgosotag acteonaget cotagaggeo agaggetaca tetestacac 1680 gagtgogotg otggtecota gosgaggoco sosogtgtgg. gosotcagga satgottatg 1740 ancaguagny signganoca aposignact tempocage teccoccage tecascactt 1800 gtoctutoon genegigagy stogggotgy goeggacene gregiggaty cogggosgig 1860 PORTORER CONSTONOTT GRADGASSTE CONCASSET TESCATEGGE ATTOTTCATE 1920 OCTODEGRAT AGGTGATGIT CCCGTACTCC GACTGCTGCC CCTTCTGCGA otgtgggoag 1980

【図12】

segradegte spraggiget setocoagoa regucarete suscocatoo teoactroak OCCOCCOCO GOCCARROCC INSTATRCAS ECACCTRCAS GACCTCCASC TTOTTOTTAG 2100 TESTITICAAT SAACTOSECS ATOSCCACST ASATGAACTT STACTGCSCC TOCSTCTSCA 2160 OCATOCOCEA GOOCTOCECO COCADOATOT BEATESTOTT CTEGATETCA ATEITCACAET CCASCCOLER STREETEROS STORGEROSTO RESTORARSO ATRICOGRAS ACCESARSES 2280 tentongoog conococcas accompart goocstcacc CTTGSTGGAG ATGTTCTCCA 2340 TEASCATUTO GATGACAATG ATGUTGCOTG TGCGGCCGAT GCCGGCGGtg ggmangoog 2400 ggggtgocat cagaggoosg toangoosto coogggangg gaggtaccos gogotoagot cagooocag gosooscagg agoagootga gootcoagtg atgggaggga atgtgoocag 2520 sytoacoggy casetysigs tegasettie occasitote categoracy otocacotor 2580 syctotyczy tyotyscyct systecoczy otycoaccet cottouctes topactucto 2640 gogocgasta oteasteeta gigagigget goccocasco egguagaset gggassessa 2700 asteceacea etgtoctgou guetocongg taccotgago cotocoogou totocotgan exagtistes asociatoro agosteguit oscitocciti ticcitossa occigangio 2820 arranages tarocottes songrappet gogggggoan agggtongra gantongra 2880 2040 areastross targesettt obctocttte pescootes sessesses eteasysses cocceggues accordaget gagoscages agoigatgoe etgagtogog etgicinati 3000 tosasoccag attgtosass stesoccocc acaccoactt cottoctgte acgtotasat 3060 manageounty appoints an amaginately managinately temperature appreciate 2120 agototoggg gatgggootg tocotgagag agcacaggac agagocgogt coccgguesg 2180 occasiones gosoctotas attospocas ottgocotat cocangadas acanotacte 3240 CHERRAGER AMERICOSE SERCOSTOGO RESOUTERES TODAUGERES SESTESSESS. ggaccagges aaggosgett occattooco tettototgo otgatgigag tteotasaot 3360 agtascagog cocagititt gagggotgao ggasticcag ocacanogat agggtotoat 3420 tensitotos ogeosecoci sessicotes tittecasat resenerote assettases 3480 3540 sagttaagta attigiccas gaccacacag ciggiaagcg gaggggcoot gosocoactg cototoateo accoaggeon gyocagoton gtgggotono otgtgguaco cotgotocto ottgozgoco concototgo noutracong aggiutoutg atcaceason tgozgataga 3860 tagacagagg agitatgigo agoccomang asgostonoc cutococcac cagocciggg 3720 esccareate rescacteco terresagot erraragase gosquetose faagsagoor 3780 segocotice gotecogety guesnager agentygy agonosigo toetagetyt 3900 tentgresga agaaaccaan agaatgggao otogagagaa agoggagoag oogggaoa successful segrecated commonted consecting transporting congunects 3960

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extocorese tectostote tettiseese extremens esterisest correspens ototgaggan gataccoscs ggosgagggo casagtgggg ctggttocag conceptoon congretto temponte agregaccoa gragasgoog gregtesgog gagggosoto satgigogag otsacgitta tattgottat ascagotost ggosottago agistitaci totosgotgi cactactaco atcaggatta tostoctoso CTGCAGTGCA CDATGATGGG COCTECETEA GECARACTIT COTECCOCTE STITUATORES TOCARDADO TOARGACACO OCCAGNOTICA CTRONOACCO CATROTONOS CCANCITCARO TACTROTAAT SCCARATOTO COSAATCAGS TOTCCotaag cogaggacet aggetcagtg occoctooto totoggasca outostoust otoscootso cogeocapay eggagesoss gtocapayay gractocogy satgagesosy gacatagage coectoscal TGTCCASCGO 99ASACCTGT AASSTACGGA OTTTETATTC GOTTETOTCA TOCTCCCCCC ACTTGGTCAC ASAGTAGGGC CCATAAGCAC OCTOCATIOCO CACCTOCOCO CACTATOCCA COCATTTUTT CIEGORAREE CERETOREET ggggatgagg cacagagoag ggcactgtgg cocatocoag gtootgotot atgitotgag tgcctggcac oggcccasac atttgttgga tggacggatg ggctggcaga gaggcaggag scotgggoto togtotosga tosaccecce sceggtggtg aggostggco agocacttos coggtacate cattigicat teasessack tigocarage agreeatiate cooggaagets tguassonso acagottoso agacacasat ascaccagt etgtgocoet gtgootogag garotoacar asscagetus cogernoaga caccecepte arrayogtar oterganusa ogłasttoto cototaggto otggagguae casgacutgg situacagug gaggosgogo tooggotgig tottoassas ogaggostot goonggonga gategigigg agagagtost ttoctarpar manatement tetromanes assecteurs acctements acteurests ctggggogg tgagoottom agagasgogt gogggtonga gggangocao cagacuttga stgecagged tingagtgeg autitatece asaggtgace aggageoutg gasgagattt osgasnysa gyasottgao compttigty tomogyatgy gatomottoe stygomycau egegggtgot gigtegggg agootgoagg ginageggoa gaotcoagag agatotatgg EXCERNING ACRESTED COLUMNICS DECEMBER CONCENTRE PARRECTER gacagootgg ascotgagto tggagotosg asgagocosg ggatgostgo agggatotgg gggtomogtt gtgtmgtoge geotttgtom otegooctom gtttocommo magggoogga contracco tgugatoost thetgeoutg gestetgact getettegan thastggest ESCORGANIO SCREEKERE STRONGERS EMPERATORO OCTADOGGO CTTTOTCCAD CTCTCSGGTG GTCATBACEA TGACACOGCT GTTCTCCTGC CACGCCATCT OCCABAAGTC ATTRACCUTE OCCTOCAGAC AACCCTOOCT GOCGATGTAG GTCTTAGCGT TCTCATCAGG SCCTASCAGE TSSTTotage ognassegts gagagaegna occosagaea gtgagoccot

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treccearca carrectra accastroca costonosca trougrams sugettorgo 6000 titgoccago igiototgga totagggioo coccaccaga caggganito coagggocca goototooto coacgiggoo cacacigotg acCITGATGT AGITGGCATT GATGTAGTCG 6120 GACCOGGGGA TETTACTOTO COSTCCOTEC ASCATCACTO GOCTOTOGTO AACCEREGET 6180 gegoggagag gaggogagat getotgegtt agccagagac toeccacaco tgggtcagac octoctymno tancargous ttotestoto assasscrats assotteose resactates 6300 goageagggg tegaggatea totetatega gagagtgagg gagttoacaa agaggttgag 6360 agcarregen ascotgoots otgetastes esessonott otgenagens tagasgoott 6420 tiseasanse espagnotot stermente tengengate socootoote sosteganes 6480 terageouty tengateasy sparscaput exproments centratouts sytematery goagootgag tgotoscaso scasaatest citerascoo trutteccot testutudo 66DO CONCINCOT TOCARACOCT ROTECAARTT OTTOACOTCC TECTTOTECA AACTOLENEN 6860 tuturutuur masstranos tongotocos assessucet ocatosusus erotososte 6720 sustantian amministration and processes amministration and processes and 6780 gotgocacca octoagotgo coosgooctg coggtoccos contgoscCT CAAACTCCTC COADAAGCCA GCCTTGOCTG TATCCTCGGA CTCCTGCTTC TTGTTCAGTT CCAACACTCG 6900 STITCTCAATS TCAGCOGCAT TCACCCTCST SECATASTAC SSCREETE ESPREENESS 6960 sgasgicago asotoagaga toccagaago toacatotga argacagoto argagaagtg 7020 EMERGEORGO LEGGOCOROS COLGROCTEC CECARSTAGA CAAAGGCGCC TEAGECCTCC ТОЛАТОСССЯ ТОТТОТТОЛА АТБОТССАСС АБВІТОСЯТВА ВОСТЯТОЗЛА ВОТОТОСЛАЛ 7140 CCACCCACTS THTAGCSTCC ACCOMMENT ENgoceastt approattos caggactiga 7200 gtotecotco etggatecco ageottguat toangueoug gongaccoto cotgounce 7260 acactetget cacagacage agoctoagos gaggotocco ogocgootag otgoottacC 7320 TOGGACATGA COTTGATOTO GOTGACCOTO AGCOGGGAGO CTGGGCCAGO CTTGGCCTGG ТСАСТВАВСА САВАЛЛОСАС ВАЛЕТСТССА ОСОТОВСТВА ООСТОТОЛОВ САСЛАВАЛАС 7440 STOCARGEOT COCCUTTOSC CTOCAGCAGO STOTCTSCOT SCCCGCCAGA CATSTOGCCA 7500 TOSTACCACO terrenergo escutorere esexteneso congeccace exernesco 7560 ctgagoagag scattoscar syaggggdog gasatosago soctactgtg tgocaggono 7620 tgogotecat gottocaset coeccucoto atteggtot otcacessos tgotgagago 7740 taggining attacoccos tittatagni gagganactg acgotgagot, gitactosci excotegago cottgactge ctotagggos agottcoaga gototattgo coaggotaga 7800 7860 gtgcagtggo gcggttacag ctototgcag cotogaccto coazetteas gccatocteg cotocagagt totacocgag citoguacoc agagoccaco totganagao aganagactg

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gonstatton anetatting agrangency ctangentus atotocytes arentageno 6040 tetrococay caregaccay agressages asserceded crargocade concurrent testences tenacaosos sautosesto sossesses cenaterest sosotococo seggaggogg sgasgacaac cocacteggg saagagtest staggeggtt ctgggggtgoc 8160 oteratesty temoteracy sympocomy stitumnosy attiguages sotgionesy B220 cagasagong enctanguou titocougan guacaccoct tipocaggag ganaciging 8280 coorsears coorsecce enganeses ogigatesus assectones seteettets 8340 remagnent gagnartene gttgtgaget ttootcagoo ongegoogae greagegeer 8400 gonastitto cancogongg gatgayayan agangayan gaccagtoty toccanocca RASO totoscoot goodgaggt gastooggoo gagagasooc cagagostoo tagoccoot 8520 rescinctio aguocaucho otserracas guiaganous caustagues seassasset 8580 gagoccotto cotoaggoes caggatgagg angtotocat goanatgtes agotgacoad eggengogon gagooncogn gggnongatg canattotog cagaggagga ggogononga 8700 casegoagag gacacagoag excetgegag enagagoete toscacacte conggetoag 8760 aggigacact agotoctaga acquatoaco cassanacco tocastacas ottocotast 8820 ottemoctoc egeocogges etgeocotoce tteettetet etettetets ttetcettgs acceangest caresteste treargress tostigueses strutustat ettoacoto 8940 carrentur terrecegto etecteteet guetgragge maggagasag agaggacatg 9000 agotttagag matotoggtg commangood ootgagtgtt goggtgggga agaggmaggg 0060 CAPTURE OR ACROSTRATCO RECORDANCE EXUTORORS ENGREENESS SCOTTERARS 9120 aggoczatou agganaggag gontocong tgggocaong ggotgonoug tgacoganto 9180 caputoteng titonatong tgoottgeoo manantattg atgoagtttg gaggoogsa 9240 totosgagag gotgosagot ggcaggagca aggittoccas testitosat ascogatoca 9200 trastgourg gatecaogge sagageaagt eggetteroo casegetgos tetrasasce 9260 ocotagogit mitoogotgo cotttactot maggagatot ggcattgaga tgoggotgot 9420 guanggagon atggtgtoat gaggagoatt cagagoccco agoccocago coatgoottt 9480 gggagaccan aggaggagto ggggaggoca caggagggas atgootggag tuttectots 9540 INDOORREGO SECRESERO SCHILACOCO ESTERNOSCH LORGATORIE OLOTOSELEN 9600 tototettie georgasen otattigaco concegnon naccontoco naccessore 9680 otggmonag gostonagtg tottggmang cattostcoc catottenco constgtong ctoclosoty tootsacsty tgotsgratty aggreeoese gyaggaageo sgrotogatt 9780 congregues sugarottic tancancega goiggootgo acquestrot grococcare DRAO" agtgeoctoc otgtotocag tgggacotgg gttgcagcgg coarggotco oggagtaggg. 9900

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oresponent thousands tootogenest territores mestanters thereofoth 0960 10020 ARROADORER TODOSERSOS ECRETERES EXPACATICA EMECOCOREO TESTECRES agggengagg stggagggta ggoagogoog gotgooagto tocotgooto octggotgtt CHESTERS GOOGOTON OCCUPATER SHATSFORES ESTECHENO COTONOUTU 10140 CACTACTORS ATCOCAGOCAR TTCAGCORGE ACTERAGETS GATGATGGTG COGTOGCOST 10200 CCTBCAGGAC ACCCTGCTGC TGAGTGTAGT ACTCCACCAS CTGTGTCAGA GTGGCAAACT 10260 TOTOCCCTCC ATACAGGTCA TAGAAATCCC CTGAGTTOTG GATCCGAATA TGGGTCACCT 10320 SATOCOCCAO Cotgosgugo socsgeoggi gagrocagto ggigosguag tagasgoagu 10380 agggongga toggotgagg tootgagtag aggonoment aggontgagt aggenesano 10440 ARRESTAGES AUTHORISE THEORY SERVICES CHARACTERS CONTECUED 10500 suganocage entgagtest toassgoots sategoaona sasastegoo sasatgoods 10560 EESTIGOSES SESCOOROOT ACCTEACEGA GASCEAGAAG TCACCCTGGT TOTTGOGAGT GGCCGGAGCO ASGAAGCTAC CGTGGACACC TCGGCCCTTG AGCAGGGTCT CTGCATCCAG 10680 COCACTGARG TCTCOGTGAA ACCACCTGRE CRECCCAGEC ARRECASTES ERRERESTOO 10740 ogiarrosco agreeccett gootopisto taccetrast greezeccet taccTCACCA 10800 TOCTOGOGGO TTCCGGAGAG GAAGGGGCCC CAGGGAATGA GGAGGTGCAG CTAGTCTGGG 10360 CARRICADADA RICTICARRICAC TAMBUCTUAR ATRICADUTOD GARTBOOGGE COSUCTUACO 10920 ACCCCCGGTG GTCCCAGTTC TGGGGCTGCC ACTCCACTgg cotggggggg cogggcagggc 10980 EXCERCAGES ASSESSORES OCCRECOCCT REFERENCES SCHEETSCHE STORTHER 11040 11100 agonougous exusavoras gosgarousy staroactes erstaureso exercocact toocsonogt ofgonztona aggotongga gontggontt gonontatgt gananoncat 11160 guagtasuga cacatututu outigoscan tonaggocoa gataataott cacguatucu 11220 saccongang giocoanggo atototocot cotocaccag eigotitian guagocotoc 11280 otgroccang otgateaces tratagoget guoseoteto etogagtene toutgotege. 11340 tgoogggogu tgtgutataa tggogttoan atgosttgtu tgattggttt dtoaccacag 11400 cootatgaes caggagotat otttatoatt ttacagacaa agaacctgag gotoaggag 11460 titianggost igootgagto catcagting tongotgosg tgoonggatt consoconga 11520 cagtooggit cohangoong tgottignan tootstacan cagoootong ggottonnag 11580 ascapagett asgagootco tigiconsar regotrocat geragoses gactastagi 11840 tampeoutty occartgotty cangagagas gggtgccoto ototaacces catatgongt 11700 tigicostgo cacigiggos gaggatges cascatgeco cigagosque cistosacat 11760 and court in the state of the s 11820 outstotoot gegegetegg gescangeto etcaccatge cteattttot cagegotegt 11880

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testotters succotesso actementat stratgotro otecctutas ttocasatec 11940 ERRCHICHEG, ROOGOCHERO CHERROGREE BREEGCOGOT CHRONIEGE THORITERES 12000 apacatatta atarottoco otaresares anactresta sotzenesao asesotares 12060 gagacactty gitototaca taccittitig antititatat gatgaguatg cattaccitt 19170 atmansates atatectesc terrescent ticteress cititagest somereure 12180 ctotttetot otatoterat gotettteca tersteteta teasogtesa stigspogra 12240 ogoagtggot cacgootgta stoocagoso titigggaggo caaggotggo ggatosogag gtorgregat ogregacosto otggoosson tggtgansoo coctototao tenanatsca 12380 namenating otgggostgg tggtgggogo otgtsatooo agoteotogg gaggttgaag 12420 12480 CHERRENTO ACTIVADOCO ESPERSOCA RELEXORES ARGORESTO ECACCACTEO 12540 actoragout ggossozong tasgactota titonamana amananama tggagoomgg tigigigott entattinig escattatgo sigitotgot toaggiages gasgapeses 12600 tiliasgoco seosastota attitoccat gatamotaca tistoatiti tagamosto 12660 mancatomat gitticocco amemnaggot titocactit totitotito tititigitot 12720 attiatitot gattitotto totosofgat gocofgagge atotgoctus tagattiett. 12780 gostgytoca gotttgacta occottoago ttggacacoa coccagagoo cogganatoo 12840 goggescoco cacgocosno gocastgago sagasgagoù cutgossago tggotgosgo 12900 gorgesonoe ogotgogot otogotosto toggagtgit cagagtecag tgogatgoot 12960 gguaotgago sagigitosa casacasgot ogiosgicos ingcoacata congeceaca 13020 agtacagett cotgetocta cacacacago etgottetos teogosegos aggatgoses 13080 cucagiones gonoscacao otonicatos atgiocecot soncecatao otggiocegi 13140 gracetooco mogostggma cataantotg soosconog sagtitiong tocatgoton. 13200 cossang cocsofgoso cotegoccos sgacectoot tecangett assoccactt 13260 metregange mattagotog gonocomena manotagot agroecotot tootsnooon 19920 13380 tegragage aggesttoot otgangegty tonegooneg agacagenot cagacages giacagotto igotatecti iggeaggasa sinasassot sectecetta cottageatg 18440 statement tecapatose titosestse segutotest tiaggostes sucapocots 18500 tgatgtagga atcetttico ocattitott atgggoggaa accagogtag ggagacteag 13560 sattaceagu gtesatgoot geteattong ungammanne tetitootte gaugosekus 18620 gataunagta gagatesags consequent tegetenego etgtaateco ageaetitgt 13680 EMMERICANCE OTHER RESTREET ACTIONS OF SELECTIONS SCHOOLSES ANACOTEME 18740 essocoteto totacansan atacanantt tagocagate testagosca cocotetago 13800 congetect terragents agatements atogetting cotgesetse togesetse 13980

【図18】

agtgagocat ggitgigoca otginatoog gootgggiga ougagigaga occigiotea meastessa stacegotgy gogosytymo ttacegotyt satococayos otitygysyg 13980 communication apprehencit gagateares etteraraco apotenzona caccatagan 14040 occostotot acterootet astoonsent totoseesse tempetteo asteascoes 14100 gatoatgoca oogoactoca gootgegoes oagagtgasu otoogtotoa saassaassa 14160 sattananc catacagato sugatosutg gigutacang sagataggea giotggango gnasscasgy casactanto tytegessyt totygesoty ototysycot cagastocco 14280 gootgacean tgagotteso estatttoco asstactgat gtgassagot gytesgtggo 14340 aggettions igoigousgo tittacatta tactattaat cotcaggous spootsotta 14400 gtaggtgits tioscoccat titagausca gnasctaggs otcagagatg tiungtautt 14480 guocasgito acasgutagt gagtaguag gtgggtanan cacagotato agauguuggs 14520 gootggtgoo ottiococca acasocatec tgcagcagac agcgtcgggg gcagatggga 14580 tergrantgo cacagocont gateganggt ggaggangan gtgotoocoo agaggoanca 14840 14700 terstoness consegned sonogonous extococute securitors agoogtuago aspocatety gotggagosa agttettoty agaggteres accordotyt agagggtost 14780 tenaggeoug gotgagaane caaggatgan atotgracon ggggaggoot ggocangeta 14820 cotggoegga mantuscoso agangganto aggganosga agantoagta gganococat 14880 czetyszyco gacagtynou ogatytycho tengecaszy gongacatyn stytynymy 14940 CARRESTORY DEFENDATE PROCESCOR COCRCERCT CECCRETACT CTRIENCOR 15000 stigiosott titotittot tittititt tittigagat ggagtototo cogotgosot 15060 carrotypay twonstgaog castotoggo tonotycano otocgootoo tyesttoney 15120 castictoot gootoegoot coctagiago teggactoso aggigogoso cocigigoos 15180 gootsettit tittitetat otttagtige geoggestit ceccetatig goosgotge 15240 tottgamoto otgacotogt gutccocotg cotegecoto commartgot gggatteone gottangoon controcong cognitation cititiotit titotitoga cognitates stitutett tistisset ististiig asigggigts asittitett titotiisst 15420 tigittiett teitteette etteettite titettett etereteitt eettitte 15480 ottottiato tittiotito tipotiocet toottotito attocoloio tittittitt 15540 tittitigno mongastott gottlatono conggotgua gigongigio monatocano tonotgongo giognocico tiggotongg tgatoctoci goctongoci tecangiano 15660 tgagaccaca ggtgoatgoo actacatoca gotatttatt agtagtaata gtagtogtag 15720 tortesture satementat atcoctetet teccceret retatienes tottessots 16780 sagigatest occasoring octoscasso igotagasot socscatest igggocates 15840

[図19]

tacoccaptat tittititas tagagatggg giotototat gitgoccagg otggittisa 15900 actorigago tosagosato ttoccootto ggootoccas agtgotgres toscagacet 15960 gogoczosto atotogiczą gitticzoti towacztost agotoczacz tegintarco 16020 tgetgiggee atgesteege tecastaggy tectotyces oggyesesut giostititi tgtttgttoc cacattaggg otttgtgcto accatotoct etgoccagag atgettgact 16140 citigateset acgicacate gocagoteet ettoctocte atoogggiet engotesant 18200 giosoctoot ogggaaggee tocotgatee etccagetag tastgiosee tgesescoes 16260 gtoactigot agostatgas gotggttoat titottitit tittititit tigagnosag 18320 gtotomotot gtogocomgg otggtgtgom gtggtgomet omtogotgac tgomgoottg acctoroage otcasgosay cototoscot tagoctotoo atagotggas coacaggacy 16440 tetecoscos osocoscots atticitati titigingag monggetoto scietatigs 16500 16560 coaggotagt otomeston igagotomag cantoninto geotoageot ocoscapteo tegentosca ggigigasco actacacong goosaggoig toistittoi gogiastaci 16620 totgaganto tgonatgato cantitatig agitatitgi toggiotgia soctoccaci agustatens ofcontensa sossassoci tetotetott ettosotoca etettacoas 16740 catottasso agoacotggt gostastage tgotcascac gtacotgttg satgastgat 16800 gonggggues gagesgiges aggresones gengateggs consectots stattggges 16860 ETECHNARIA ETTERCTERA CETHERREEK COTTCGCERE TERCOTOTRA OTGOTETET 10920 traggigite igagotacco arreguecas stociassos sussessos songgascoc 18980 agaggotgan agtagoaatg castgyagag ancagataoa capttatoon gagganaatg 17040 ttenegagge agregagges catgagtene coggregato tomomouts ogggangggs 17100 augitosing genocygens congogong gongogong singosotos cyticacacs . 17160 ogtograpty agoatoanon angocotoco tocotocana actmacagea gautgeracu construction constructs of CACCOGGAC ASCATCTCTC TOTAGGGAGG GGGAAGCCAC 17280 DOCCERAGEOC COCAGEOGEC EGGECTOROC CAGOSCORCA ECTOTECROR GTDGCCCARG 17340 CCAGGTGAGG AAGAACCGCT CAGTAATGAG CCAGTTOCTG CTCGATCCTG CCTGCCAGGC 17400 CAATEGOROT gosgugaces accordigant astagtotco aggaseauge caugingsgo ERECTERO CERENCORCO CHCRECERO ARCTECCCE CHCCCCCRR GOUGGESTRO 17520 agoaggooog outgoiggto cagggoogoe ticcoccagg agaggagag coacciggag 17590 ctrosoctes agotomagot gacagagoge oggretatte egtaggegan cagacoagen 17640 agtasocoty gotgaggaag agosaconat gggggaaacc totcogtagg ogganocan 17700 aggroctuag gasantocot sacagoocag theeteagag saggagetso cocotytyca 17,760 EXOCCACOOR OCCARGODO ESERGISCOO ROTOCAGOOT OTCARCOTGO COCGETEGRA 17820

[図21]

ATOCTOTOCO OTOGOTOGIT TOACCEADAD CTCAGTOGGO TEGATOCADA GACCOTOCTO AAGGOCCEAG GTGTCCACGG TAGCTTCCTG GCTCGGCCCA GTCGCAAGAA CCAGGGTGAC TTCTCGCTCT CCGTCAGGGT GGGGGATCAG GTGACCCATA TTCGGATCCA GAACTCAGGG 180 BATTTCTATE ACCTETATOR ACCCEAGAGE TTTGCGACTC TOACAGAGCT GGTGGAGTAC 240 300 TACACTOARD ADDARGETGT OCTGCARDAD OGCCCACERDA COATCATCCA CCTCAADTAC CONCTRAACT GOTCOBATCO CACTARTGAS ASSTCRITADO ATORICCACAT STOTEGORGE 360 CARGOLAGAGA COCTOCTOCA GOCCAAGGEC BAGCOCTOGA COTTTCTTGT GCGTGAGAGC 420 CTCAGCCAGO CTGCAGACATT CGTGCTTTCT GTGCTCAGTG ACCAGCCCAA GGCTGGCCCA 480 540 SECTOCOCCE TRANSCICAC COACATOAAS STCATETECS AGEOTEGACS CTACACASTS 600 GATGOTTTGG AGACOTTOGA CAGCOTCACG GACOTGGTAG AGCATTTCAA GAAGACGGGG 860 ATTOAGGAGG COTCAGGOOD CITTUTOTAC CTGCGGCAGG CGTAGTATGC CACGAGGGTG AATGCGGCTG ACATTGAGAA COGAGTOTTG GAACTGAACA AGAAGCAGGA GTCCGAGGAT 720 ACAGCCAAGG CTDGCTTCTG GGAGGAGTTT GAGAGTTTGC AGAAGCAGGA GGTGAAGAAC 780 840 TTECACCAGO STOTOGRAGO SCASCOGOCA GAGAACAAGO SCAARAACO CTACAAGAAC ATTOTOCOCT TTEACCACAG CCEAGTEATC CTGCAGGGAG GGGACAGTAA CATCOCCGGG 900 TOCCACTACA TCAATGCCAA CTACATCAAG AACCAGCTEC TAGGCCCTGA TGAGAACGCT 960 AAGACCTACA TOGOCAGOCA GGGOTOTOTO GAGGCCACGO TCAATGACTT GTGGCAGATO 1020 SCOTTOGGASG AGAACASCOS TETCATOGTC ATGACCACCO SASAGGTESIA GAAASGOCGS 1080 AACAAATECS TOCCATACTE ECOCGASGTS GECATECAGC STECTTATEG SCCCTACTCT 1140 STEACCAACT GOGGGGAGCA TGACACAGC GAATACAAAG TCCGTACCTT ACAGGTCTCC 1200 COGCTOGACA ATGGACACOT CATTOGGGAG ATCTGGCATT ACCAGTACCT BAGCTGGCCC 1260 BACCATOGGO TOCCCARTOA ECCTROGGOT STCCTCARCT TCCTGGACGA SATCAACCAS 1320 COGCASGAAA GYOTGOCTOA OGCAGOGCCC ATCATCUTGC ACTGCAGOGC OGGCATCGGC CHRACAGECA CONTRATTET CATCEACATE CTCATGEAGA ACATCTCCAC CAAGEGCCTG EACTETEACA TTGACATOCA GAAGACCATO GAEATGETEG BEGGGCAEGG CTCGGGCATG 1500 ETGCAGAGG AGGOGCAETA CAAGTTCATC TADETEGCCA TOBOCCAGTT CATTGAAACC 1560 AUTAABAAGA AOCTOGAGGT OCTOCAGTOG CAGAAGGGCC AGGAGTOGGA GTACGGGAAC 1620 ATCACCTATC -CCCCASCOAT GAAGAATGCC CATGCCAAGG CCTCCCGCAC CTCGTCCAAA 1660 CACAAGGAGG ATGTGTATGA GAACCTGCAC ACTAAGAACA AGAGGGAGGA GAAAGTGAAG 1740 AAGCAGCGET CAGCAGACAA GGAGAAGAGC AAGGGTTCCC TCAAGAGGAA GTGA 1794

[図22]

N				C
8H2	Γ	SH2	PTPase Domain	C-terminal

【図20】

grandento otongetage gitagecoot augrospert tageocome osgracosta	17890
ESTABLISHED Executable spacescook transledge absenced fortheribe .	17940
teggestigt orgatoogca agagaotgao accampute agtoscaggo goatttatta	18000
tigtotggas ostonaggoc titcotcoco iggcagiggo sonagggagg gcomactoto	18060
aggaggoggo caogotgoca coagoagoag goocatgagg tagcagggto atgganggoa	18120
gasacegogo ottosgotty ootgaceggo tggogatoto aggatoctgy gottogtagg	18180
sotignocan gogagoanso tisaggacan otgonagang gagtggangg toascootto	18240
tootsaggad cagogtgoot sagmoscotg gagggggtgg tagtotungo atganotgot	18300
occoracter oterscanot attitocasa canaccost scannoctot atocomists	18360
stitignates tencetities totacecities goaceacities atter	18404

【図23】

MYRWFHRDLSGLDAETLLKGRGYHGSFLARPSRKNQGDFSLSYRYGDQYTHIRIQNSG
DFYDLYGGEKFATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSG
GQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRY
TVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYYATRVNAADIENRVLELNKKQESE
DTAKAGFWEEFESLOKQEVKNLHORLEGQRPENKGKNRYXNILPFDHSRVILQGRDSNI
PGSDYINANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVE
KGRNKCVPYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQ
YLSWPDHGVPSEPGGVLSFLDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENIST
KGLDCDIDIOKTIQMVRAQRSGMVQTEAQYKFIYVALAGFIETTKKKLEVLQSQKGQESEY
GNITYPPAMROVAHAKASRTSSKHKEDVYENLHTKNKREEKYKKQRSADKEKSKGSLKRIK

780

1020

1080

1200

1260

1320

1380

1440

1500

1560

1620

1580

1740

1800

1860

1920

2040

【図24】

autocotott goaggigtoo tisagittgo toottegio sagicoto asgoccagga 240 tootgagat congoctgt caggonagot gaage otg tttotgo contracoct gooscoccat gggcctgotg otggtggoag ogtggogoc tootgagagt tggpootooc tigigoosot goonggggag gaanggoott gatgitoong constantes atgacotgi 420 gastiagest tagiglossi stottamings octasesses occatatote ettocotest 480 tocototgoo titocaggoo coatococot gascagotoo tocotatggt cotggotggg 540 cotasocote ooccagegoc tascoctaco tesegotoct cocottocco Esegotest tgagaggotg gagtgggtoo otoagggooc tgagtgagtg agcotgoaca gggggtacot 660 cottototga ggasotgggo tgttsgagat tttccttagg ccotttggtt tomoctagg 720 gagaggitto oppositiest teotottoot osecongest taottootes totattooog teccosateo como to tgtosgotte agotocaggt ggagotcoag gtggotcoto 840 ctotoc gg ggesge go cotggscosg cegggggc tgotgtecte chotttggg gotgoaggga agotggo tgtggg gt ot gggccag occ cocca cotgtocttt 950 tootggagao tattagtoos gggtttgtoo otgoegtgoo ATTGGCCTGG CAGGCAGGAT MAGGAGGAA GTGGCTEATT ACTGAGCGGT TCTTCCTCAC CTGGCTTGGG CCACTGTGCA CASCTATECC SCIENCICAS COCCECCCC TECRECOCTC TECRETAGET TOCCCCTCCC .1140 TACAGAGAGA TOCTOTOCH TGGgtsagto chegcacoa thegggtoco agtotoctgt tagttttega gegagggagg gotttgttga tgotcactor autigtgtgtg autigegtg Matolgo otgoocts cotstitc stocctats acticount companyst gtgaggacco of gotoact catgotocto tgooccotot ttsacatttt cocctggaca agtgtgtato tgttototoc attgoattto tacttocage ototgegoto otgottotgo ctootgotta ggacotgtoo cootgggtag otoscascac otossacata goagtoagag goosco asgrocotco cantocago casetteto cacttocas sestesgact ttegtopest ettettisti teettiseet tooottiege etgestesti cattossess gta tgttg agoatotatt atgoacosgg tgotgtttas gatgotggta etsotggagt gasosogaca gacategtot ofgototos gagottaca thooagtggg aggitacaga cassat escocentes attgentest tycapattot cagasgisti amcagazas tagacagoot tego meets tagteettos cacctetest cocagosote tereserote segmagage stigotigas cocagagit tgagaccago otggocasta tagigagaco otgiototec sesseineg sestingotg getgigging cacemitoct giggitcosg ctatgagag gotaaggtga gaggottgot tgagootgug aggtosaggo tgoagtosgo gatgattgon constgones ocagootggg macagagtg agacottgto tonamenana 2100 sasasassa gasastgaso cagottosta tgotagonag tgaotgagtg tgoagatgao 2160

[図25]

【図26】

GGGAAGGGTCTACCCTĆGGCĊGCĊG

GGGAAGGTUTAUUUTEGGUEGUEG

【図27】

ttatttagtt gigtttagig ingattagat-gitattattt enatteteog ggstageggi tigiatgogt titgitiggt comingitigt tiagitaging geographagi timigitiag 120 attettatit tiitiaggig tittaggtao gttggttitt aggagaaggg tigatitttt 180 stitititt glaggigttt ttaagtitgt togtitggti sagtitiacg sagtitagga 240 titigagato gitagitigi taggiangit ganggogita tititgicat tinivatiti 300 strattitat gegittigtig tiggigging ogtgetogit tittgagngt tggittitti 360 tigigitati gitaggggag ganaggtitt gatgtitteg stantastan nigogtitgi 420 gattlagtit tggtgitagt tttttgcgga tttgateatt tttatittt tttttttgat 480 titittigit tilitagett ttatittttt gaatagittt tititatget titggtiger 540 titestitig tittegggtt teettiisti tgaggttiti titititit carrateggt 600 tungagutte gagtegettt tittagogitt teggigggig sgittetata gegggtatti 660 tttttttga ggsattgggt tgitagggat tttttttagg ttttttggtt ttogittog 720 gagaggitti titiatiggi igittititti tagitagggi tattittigg ittgittitt 780 tattiamint ttogtogttt tgttagttig agittiaggi ggagttiteg gtggttittt 840 tittttoggg-ggmaggoggt tttggattag taggogggtt tgttgtattt togtittggg 900 gttgtaggga agttggtogt tgtgggoggt ttogggttag titogtitte titgttittt 980 ttttggagat tattagttte gggttigttt tigtagtgtt ATTOGTTT6G TAGGTAGGAT 1020 COAGGAGGA GTGGTTGATT ATTGAGGGT TTTTTTTAT TTGGTTTGGG TTATTGTGTA 1080 TABITETETC GITCETITAG TITCETTTIT TECSOTTITT CETCSTSSIT TITTITTIT 1140 TATAGAGAGA TETTETTTCG TOGEtaartt togggtette toggggtitt agttittet 1200 tegittigga gagagagag gittigitga igitiatito gaogigigig aacqigagig 1260 ogattigtog tigtilitgog titgiliting gittitlatga attitititt tingtanggi 1320 stgaggattt toggittatt tatgittitit tgtttttttt tteatatttt ttiliggate 1380 agtatgtatt tgittittit attgtattit tattitlagi titteggitt tigititigi 1440 tttttettia gyattigitt tittgggteg titetaatst titeastata giggttegen 1500 gitatiogog maggittitt tacgittagt taattittto gistiitita sisitagati 1560 tiggtilist tititiigit tittittatt tittititt, tigtalisit tatitostes 1620 gteogtette egtetttett etgtettege tettettee getettegte etettegeet 1680 gantangata gatatgatti tigittitiac ggagittata tittagiaga aggitataga 1740 togastesst satttastes attgratist tgtegatitt tegasgtatt sostegassa 1800 tagatagitt tagtogagitg tagtogitta tattigigat titaginitg igggaggitg 1880 aggogagagg attettigag tittaggagti igagattagi itagitaata iagigagatt 1920

tigititiat assensing austingtig gatgiggigg totacgitti giggiiting

[図28]

tistgragas gitaassisa gaggitigit igagitissa appitaassi istagitaso 2040 gatgatigta ttattgtata tingtitung ogatagagig agattitgit tiasas 2100 assessess generations togethers titlestes tratingging totaggings 2160 attattagtt ggagggatta gggaggtttt ttogaggagg tgatatttga gttgagatto 2220 swatgagger geagaggagt iggitatyiy sogiagigat taagagitaa stattitigg 2280 gtagaggaga tegtgagtat assettttas teteggaata astasassas egatagtete 2340 ttogtgetes eggettties tegescopes stegestiat egtesetter ettetetter 2400 ngttaggatg tigasagiga asstitgacg egatgaggig gogtacgitt gigatitieg 2460 talttiggen ggtogonggg ggangatigt tigngitter gagtitamen tingtitggg 2520 testatagag agattitatt titattassa assastatig ggiatgatgg titasgiatg 2580 testestiti estestitus sevetteses tessessett etiteastit essestities 2640 gattatttig ggtastatag ggagagattt tatttttatt sogattsoga ttattattat 2700 tattastass tagtiggate tagtestate tattigiest titustiatt tegnageite 2760 oggtaggagg attettigag tinaggaggt ognogitgia gigugtigga tigigutati 2820 gintittegt tigggigete enginegatt tigtgitnen menanassen menagagegg 2880 2940 THERESE STREETED STREETED STREETED SERVICES STREETED SHEETERSTE OGREGATION CONSTRUCTE CONFIDENCE SANGERS STREETHON CARROLLES nagtgatait tagtoganag anganaggan agammangan anagtgatan toggtogana gnaanaagaa mangigataa toggiigggi atggiggiii aagiiigtaa tittagiati 3120 tteggaggto gaggtaggtg gatteogagg ttaggagttt aagattagtt tegitaatat 3180 3240 getgezettt tettitaatt sangetetaa assennantt agetigetet agtgetgogt stitigings titiagitati aggregatig agginggara attgitigas ittagunggo . 3300 susuetteta greatrogas atteogram tetattitas tituagista sormanasa tittatitta masannansa sannaganna gannangiga tantiigitt atagagiati-3420 geogagitty teggitgestis gittittegt titgitgatt titgititti ataliiaigt 3540 tigittitet titogietat attitettat tetogetitt atgesteree tittigites stittitest tittgattto stitstest attitittet taggiagitt egitagetti tttttegigt agattitatt ttiggittit tagittggit tigaatgatt tiitatagta gggtttttat ttittegest satttigttt tegttatetg gttigtttac ggtteggtat tgittatgig gattitgigo gigttattit titgittige titatgitgi tittggggga statititit tittattitt tetteteest tetesteete titattitet tiettitosa 3840 ogtigitigt igtagisigg tigitggggg assganist eggittoggo gittgategi ogtgitttet ttattittt atttettagt tigigatitt gagtesttet tientettit

3000

3060

3360

3480

3500

3860

3720

[図29]

tgagttitag tititgitti tamastiggg tgomtamist tisitangin gegitggitt 4020 goggattant agistastet assagtiegt agistigsse titigitati tettegtitt tisistiagi sittigggasa tattettaag titattigit aggoggggat titigaggitt 4140 agagtagttt tagasttitt tatagatiat titigtitigt tigogttitt agattgilia 4200 tttttttgta ttattatiga tittgatite tatesttttt antitttitt titttensag 4260 ggagtttiat titgitgitt aggtiggagt goggiggtat gettioggit tattgtaatt 4320 titiatittit gagaagitgg gattataggt tagtagagat gggattitet tgtgttgttt 4380 agtiggitto goattitiga tittaagiga tilititatt toggittitti maagigtigg 4440 gettetagget gtaggttatt gcgttiggtt gtgtittat titttgaggt agggttigt 4500 titettetti agetoggati atagiggisi sattaiggit teligiagii togattetti 4580 taggittagg ogsittlitt stittagtit titaggistt tagggitate gangistati 4620 attotalitig gitesattit gtettittig tegagetagg gittilitag gittittagg 4680 ttgittitaa attiggigit asginaltin itagittist tittataang igitgggsit 4740 stargogica attoticogi tigattites titttettit tettititis titttasses 4800 satatititt tittignat tattaggiat trattitigt nattittagt tilittaggi 4860 tigittitet itatuagasa siguegasa teatititat attatuaget igittuaget 4920 ttanatgaga togigtatgi ganagigati tetanatiti etattatgit asgginages 4980 aggingitit itaitittt gitaanget agtegenett gistititig titgagitti E040 mittitiest tiestettit tiesessest titistitit titetevett ervanusest **5100** gittagitag tilttitigg gigtogagit matitititt tottmaging gittangitt 5160 terangenet ettiterest taggetetas terecettte etetteneta teratityan 5220 etitogiste igglingati inigititat gogiggggat. giginiogga itagginigi 5280 gistagging atalogates teagetetet gististest icinistate tititatite 5340 ogiatgates giaggitate igigiagrat ingenegits tattigiest ingetatete 5400 gitategatt geogagitie titetigaat attigities tettagetat ogiatigat 5480 ttigaatatt togagatgag ogagagogit agogggtgit togogitgia gitagittig 5520 tatgtgttit ittigttitt iggogtiggg ogigggggit togoggatti toggggttit 5580 suspicutet theautions suspication and treat at etactions titatteret 5840 agattitita gugtattagt gagagaagaa aattagaaat aastumaata aasagaaga 5700 segennegig generatitit titiggggge asstattgat gitigatgit titesseatg 5760 stantglagt tettatgggs senttagett tettgggttt mesettlitt titttttat ttenegtega atatgiotee tettetees tettenetat atesitiggi titettitit FRRO tttittitit gasatagagt titatigigt igitaggitg gagigiagig gigigattit 5940

【図30】

ggitogtigt estiliggit tittgggitt segigetitt titgitites titttogsgi 6000 egitgggett steggogitt ettettetgt ttegtteett tittigisti tittegingeg 6060 aggregatitt attateties tiegratest titgatittt teetticate attositest 6120 6180 titiggittit tasagigitg ggsttatagg ogigagitat igogitoggt taatittace tttatututa tttatgiman tagiatitug atagagatan agagtttttt ttgtatttta sengittitt agnestigit titagitegi atetitetti tietosaggi asigiaigit 6300 tettetetam tettteesam gytatgtega gasttaagts tttttttiing tittgittti 836D testiatite etitititit tiegresser tistiasist sigititite intettitt 6420 gitgogitgt tittittogt titggittgg oggigitgst gitigiatit ggnatiates 6540 ttagttatgg tgaggatttt gttttttagt ttttaggaga taggittitt ggtgggagtg 6600 tiggggtagg gtagaggtit agggatagga attaganogg attictgtig atagggtigt 6660 tinggettat gitgittatt tittitettat agteriates ataasiteta inteligett 8720 agaggagget attititit titigiangin tigginaggi titantinit agtititigi 8780 tittatggta gtittitigg stauggeggt titlestitt tgittitiga agtittungg 6840 gitggigist eggagitics agistiggit tiggostogy attgittggg titgostitt 6900 agististes tigatitett satesettia satestatti taastititt sastitiess 0393 ttttttgttt giesestgat easgetegtt tttgtittet eggettgigg tgegusette 7020 stingstong gintgigeno gitatteteg tategogitio ggintitagi eggettiati 7080 ogatgatagt igitatogtt attattetta ttagogtaga tiagggagga tigogtamas 7140 stagticate sassasses assistoris exatostits spitostate occupantat 7200 tettiesett temestetet essetatete tetettitte timietetet tetletetet .7260 etsisstett steittigs gittitesti stagsogtet gereastess titogittit 7320 attittagig tietiligit tigittitti tittitgitig tgittissas ogaşeaşlat 7380 sogtgagttt ttttsagggg toggtogogt ttttttligt tttogtttig toggttgttt 7440 TARKTHARTE BASTOSTAST TITASAATTS GEATTATOGG BOSTOSTOAD GOGETTOGST 7500 ATTROCARTY STATTTRAGG TITACITTITE CARTTITITE TITELITAGA TIACITETAT 7560 TITITATTI TITGCSTTIT TITTITITC GGAAGTITTI AGGATGGTGA Ggtmaggett 7620 tettatttao getagataye aggtaayegt gittggigtt tacgegatit tittitatte ttttgtttgg gtogtttagg TGGTTTTATC GAGATTTTAG TGGGTTGGAT GTABAGATTT 7740 TOTTTAAGGG TOGAGGTGTT TACGGTAGTT TTTTGGTTCG GTTTAGTCGT AAGAATTAGG 7800 STGATTITIC STTTTTOGTT AGREGATER ELLELLERS attlograte ittlegitat 7850 tittitgigt tettiaggit tigamitelt tetititggt tittogiggt agigtigett 7820

[図31]

tttogtttat tittitatti tigattitta inititilat tittattigt attiattist stitutatet ettitatit agestittas tosattitie titititati titattitie 8040 tatogetteg tittatogtt tegtettite taggetgoeg GATTAGETGA TYTATATTOG GATTTAGAAT TTAGGGGATT TITATGATTT GTATGGAGGG GAGAAGTTTG CGATTTTGAT 8160 AGAGTTEGTE GAGTATTATA TITAGTAGTA GOGTETTITG TAGGATCGCG ACGGTATTAT 8220 TATITATITI AAGTATICGT TGAATTGTTT CQATTTTATT AGTGAGAGET geggetttog 8280 tattitogit stittinggt aggregateset oggittitat titugatest taggregates 8340 aggagating tagioggost intitatiti thatititi thittitigt attagitums B400 gtttttaatg tttlttttt tigttgittt gegattiggt gttttagegt tteatttett stitititia ittestitog aggregitat aggregitet itogititat tiogggagit 8520 ttestortte taetttaget titatteras stargement tattettest gettagtate 8580 togtgtaggt tagttttgtt gttagmangt tttttttttt tigmantoga gtttgttttt 8540 tttogtttat tttttattit agtatetett aggategtga ggasttgata ttggggtgas 8700 gatggggatg astgittgit asgatatitg atgittigit tiagtogitt ogtgggatg gettigttit giggggitas singgittito ggittsasta gagetinitg agagtacgat 8820 gigangight tattigigia angightita ogitatitog gatelagagi astattitag RRRO statttttt tttstsettt tttcretttt ttttstsett ttttstsest stesstisse 8940 syttement tittenstett tittetgete tietgetitt tittagiegt ogtatitten 9000 tgitagatit ititagagia sagggtagag gastamogit agggggtitt tatatgtatt ttigggites gtogettigt tittigtogig gettitigte titetggeto ggitetiges 9120 atgatoggga attitgtitt tgttagtitg tagtittitt gagattoggg tttttasett 9180 gtattastat ttitggttas ggtattgatt gametttaga gitggettog gttacggigt 9240 agtitigies titatities agaittitit titiesatos stititita agaittitit 8300 ttittitete agtittatat gettegitto etgittatti titgittitt ttitittiti 9360 togtesteit tagggggtit tiggtatogs gatittitas agtitaigti tittittitt 9490 ttitgttiit agitaggagu ggaggaogag ttgattagtg ttiggaggtg gaagagaga 9480 9540 gtagugttit aggaggttit tgtagaggag gitgaggtti gggittaagg agaagagaga ngaghgagan ggmagggagg gtagtgtogg ggogggaggt tangattagg gaagtogtat tagaagetett titagastaat togettetaan agttagtatt attitigaat tiennastat 9860 gtgegaggit tilititite ggittigtig tgtttitigt titgttigte ogtttttttt 9720 tttgogegaa titgtatite titttoggig gitttgogit tittgiggit auttigstat 9780 tigtatggag attititat titegggtit gagggaageg gittagitti tititogita **P840** tttggggtit tagtitgtit ttaggoggtg ggttgaagta gtttagtggg gitnggaggt

【図32】

titaggggett titoggtigg agtiatitito gegingeget gagatgggtt gegatagatt 9960 agtitititi tittititt tattitigge gitegesent tigitogitt tittitiggit 10020 titgygitga ggmanttita tastittatt tittattitt titttegneg gagtittgig ttttttttat teogtegtti tttgiggggi tgggtittigt ggggliatag ttlittitig 10140 assonagast stattiones gamessettt agittigitt titettiten tagttittit 10200 assitogilt gastiliggs tittititta gigatattat tiagggiatt tiagsattit 10260 tistatisti tittittias tesestisti tittioetti tittiseogea soristitia 10320 10380 ttogetttgo gtittittt giittiggtt titgitgggg tatagtitta ttittisogg agatttaitt tiagittitt ittitissat attitgaata itgitagitt ittigitiit 10500 tagagetree tittegetto gangitoget tagastitte gagettagen testitgant 10560 ttgggeggte geggtigtes agagtigtes tegegtiett giettitegt tigggteste 10820 gagtttigge agttigttit agagtlagtt auggstitta ggtiegiges testagtite 10680 gogitagitt tittatitat assatggggg tastatista titagilitt agtatgittg 10740 tgagagattt aastgaggig giggattigg asgistging ogtagigitt ggintatagi 10800 aggigtitga tittoggitt titttigiga eigititigi tiegogitti tittigiggi 10660 ttgggtttta tttttttgg ogttgttttt tttaggTGGT ATTATGGTTA TATGTTTGGC 10020 GEGTAGGTAG AGACGTTGTT GTAGGTTAAG GGCGAGTTTT GCACGTTTTT TETGOGTGAG 10980 AGITITAGIT AGITTOGAGA TITICOTOTTI TITIGTETTIA GIGATTAGIT TAAGGITTGGI 11040 TIAGGITTIT CHITTAGEGT TATITATATI AAGGITATET GOBAGgtang gtagttaggo 11100 11160 11220 tigtingent timestites guttergrat tieggrages scattinget titatemets. Ettastite ettitita eggregacer tatayanten orenttiona gattitcoat 11280 AGTTTTACGG ATTTECTEGA GTATTTTAAG AAGAGGGAT TTGAGGAGGT TTTAGGGGTT 11340 TTTSTTTATT TGCGGTAGgt toggggtagg tttagtigit tttttatttt ttttgagtig 11400 ttttttagat gigagittit gggattitig agtigtigat tittogtitt tittlattit 11460 AZTOGTATTA TOTTACGAGG GTGAATGCGG TTGATATTGA GAATCGAGTG TTGGAATTGA 11520 ATAAQAAGTA BGASTTOGAG GATATAGTTA AGGTTGGTTT TTGGGAGGAG TTTGAGELEE atesteensa topptasset tyeistastt sagatestes taposettte seettittase 11640 oggatattit tittittiig titattilig titligatit attitacgig agittiticg 11700 11760 etagaigiit tiitigagag tigatgiila tilittiati tatattitag AGTTTGTASA-ACTAGRAGA GARATTEG TATTAGCETT TEGARGETA GEOGETTAGAG AATAAGGETA 11820 AGAATCOTTA TAAGAATATT TTTTTTIgtg ogtatttagg tigittintt tatttaggat

【図33】

stocktitte tittestist tittititst titsterett titettitt soutseene 11940 gagttattit titatattit tiatagagti titilititi tisasaggit titatititi 12000 ttagangigt tittitatta ttagtaggta ggttgtttit tgttittant ttittigiga 12080 attititat tittittata tagatgatti titattitig tigtitatag tittitogtas gittiniggi tittgagett sgantggitt gitagittag gagggittge tittaggigtg 12160 gigagititt gettaetita gattatitog tittititto gittatitit agTTGATTAT 12240 AUTOGATIAN ATTACTION SOTTETANA TRACADROCA CORRESTITE AUTOROCIDA 12300 AATTAYATTA ASettagtag tgtgggtteo gtgggaggag eggttgggtt ttgggaattt 12360 ttigttiggt geggggnitt tegettinge getegtigge tessestogee gttgettitt 12420 tgtetgggtg agggtggteg tggttteggg tttgtgttgg gtteeggggt ttettgtttt 17480 EMERICAN ACCOUNTS AND ACCOUNTS AND ACCOUNTS ASSETT 12540 AGATITATAT CETTASTTAG EGITGTTIGG ASGITACSGI TAATGATTIT TEGTAGATSS 12600 COTOGTAGGA GAATAGTCGT GTTATCGTTA TGATTATTCG AGAGGTGGAG AAAGGTCGGZ 12660 tagggogitt titititito gialiogiti togigitigi ggitatgita tiaagtogaa 12720 gagingting atgitugget agaangaget titagagat agagituggi titigitgag 12780 agatteegen tiagtenten entitomatt atatamoste attittaest tittetatet 12840 stittigggt tilittigagt tittagattia ggittiaggt tgitittitt tittitiatti 12900 tigititati igiligiati inggittitti tigittititi igititaing attititigg agtitgitti tiettitgia agtittitti etalagiali tilligiglig italigangi 13020 gettitatto gigatatasa tigggitaan tilittiiti tiigasatti tilitatast 130BD ttttgettet ttttgggate angiogiati ttseggitte gietttangg tttggtggtt 13140 tttttttgat togtatgttt tttttgangg tttatogttt ttagtagttt tagtittttt 13200 egyttitteg tittititig talangilia tittilgita gganatgatt tittitatat 13260 tettttigtt tegtegetet tiogittitte mageitategt oggogogite titttittet 13320 goattteget titettittt tieggattte geggengsat teogtititt tiegttaogt 13380 ttittagege ggtgtttttt teggttatti gtittigiga gittitegag gtstaggest 13440 stagattere tettattigt ettisteaus tigieteett tetateetti oggegentaat stitettite stancettie tiganteata asoggateta toggiguagi settestas 13560 gtittattet tigttagige tigattigag nogagagitt aggittitig tittitigit 13620 agittattog titatitaat assigtites stocktetta satatitasa statagasta -136B0 genttiggen igggitates igtilitätti igigitilat itilattoga ittitittit 13740 THE TARGETTITAL ATTRETTORY CONTROL ASSOCIATE LASCOTOLLY VICENIA ATTRETTORY TITTGTGATT AATTGCGCGG AGTATGATAT AATCGAATAT AAATTTCGTA TITTATAGGT 13860

【図34】

TITITOGITG SATAATgiga giggittita ogittigtti tattioggga gittittiti 13920 gentitetit tittittes torestages teaseterat sasstatito sasasassas 13880 BESETATION TELESCOPE BESET TOUTH TOU 14040 ASTATTIGAS TROUTICEAT TATGEOGRET TRACTEAGET TOGGGGTGTT TITAGETELT 14100 TEGATTAGAT TAATTAGCOG TAGGAAAGTT TETTTTACGT AGGETTTATT ATGETGTATT 14160 OTACIstance atentestit tentestast astratasti succestinas tattettons 14220 14280 tgitatgagt tattataagt astateaacg ttagttogta tattgagtgt tittogitta tittoggitt tittigggit tittitatggi tittagaatti teggitggato giggitggsa 14340 tiagittiet titigettitt tettigigge tettititit agagtititt toggatgiat 14400 tettiquitt mettigite metategegg eggagitogg gattingtig tiggitaggi 14480 tteagttagt teagsteary toppgtaget etttateste sgittstett togettettt 14520 ogittititt ogggettite tittettegt tittititit aggaetatit atgagetate 14580 tettititat tittititt tetttatoge tagtogtage etttogette tetteteatt 14840 tigittitt tittagittt titeggiagt gittlattit ggittitagg gitgiggg 14700 getgggigat gittittigg ggitgtetat aattittitg titalitatt ogiatgitig 14780 testioneer attitients serietores stemments toomseemes toomsettit 14820 14880 stageteset ttattemett suttigettt mustgenten gagetaging statagestt ttttogttta ttagtigtgt ggittiggat asattatita attttttaa titttagtit 14940 ttttattigi sassitagga tittagggtt gtogtgagae titsatgaga tittatogit 15000 giggitggme titiogitegt tittessaat igggogitgt tellegitte ginetitate 15060 tingstages establishes terrestite titigities stitititi stillities 15120 tegottitag gittgogace gittitggit tittittitt tittinging figitigitt. .15180 tergatages tangtogett genttingag stattitogs teggitetto sessencess tittgttitg tgttitttte gggataggit tatiltogag agitatittt ttgtttatit 15300 gitatetete tetitatete tittitgeas gittiatest tittatites mogitatess 15360 assessment statement thattities testifiest tissesties starogerat 15420 ttagggstatt agittgttgg gittagtiga gugtgggttt ggggttittt igaggittgt 16480 tigittagge tigggenogg egegenetti titetigini igittitiig egittizige 15540 ttttgtgttt togtattttg ttgttttagg gttattttt ttttgaogit agggtttgaa 15800 gganaaggga agtgangtta tgitgagaga ogtittatan ttittitung gagangongg 15680 gagggittag ggtatttggg agtoggtagg atagtggtgg guittegggg tittaggitt 15720 ticeneries eestagitat thattagene tengeneton gogogagene tenangaran maggatgets stagtteggs agttegoett agtatogtag agttogaggt ggagogtett 15840

[図35]

totgtagagt tgggtamatt tttottatta tligttoggt gattitgggt stattttttt ttattattag aggittaggt igtititigig gigtitgeng tignagitga gogttgenta tittittit oggggagggt tigatiggti titgatagta tittogtitt titttag@T 16020 COGTATOGET CUTATAGGTA TTATTATTET TATOGATATE TITATGGAGA ATATTTTTAT 15080 TAASGatese egetattive prettteres etgeresets astastitit cretaticat 15140 ttetgtttgg atttgaggtt tgattgtttt ttatttegGT TTGGATTGTG ATATTBATAT 16200 ATECCERCE ADATECTOR TORROTTECO ATECCERCE COTARACTITA TRABARRATT 16260 ADSTRUMENTAL AND ATTACHTA COTTO ATTACHTA COTTO ATTACHTA TO A CONTROL ADARDATED ATTACHTA TO A CONTROL ADARDATA TO A CONTROL A CONTROL ADARDATA TO A CONTROL A 16320 GGTTTTGTAG gtgogtgtag agtaggettt gengerener genettgtag tetagrater 16380 gtgttattig gtittgtigg gattettatt tttttattgt ttttttgttt atagTCGTAG 16440 AAGEDTAGG ACTOGGASTA COCCATATT ATTTATTTT TAGTTATCAA CAATETTTAT 16500 GTTAAGGTTT TTCGTATTIC GTTTAAgtge gtggttttge ttgttettgt toggtettta 16560 ttitttigtt tigitlagit ogsittitat tittiggaga ggstasgigt igtagitggg 16620 gegattingt titeogitte gettigetti tiettitti tettiatese tettitites 16680 gigittateo gigigggitt tigitaggie tiagiagogi attogigist gagatetasi 18740 tittettitt taggagtite gagtitagig tagggatogi gattgogita titgigagmo 16800 suggestes sacrepatic tractutogs attitute gitettiti gattigiati 16860 sattgittgi attigittit tigisitogg tigisgATAT AAGGAGGATG TGTATEAGAA 16920 TITGTATATT AAGAATAAGA GGGAGGAGAA AETGAAGAAG TADOGETTAD TAGATAAGGA 16980 GAAGAGTAAG GETTTTTTTA ADAGDAAGTG AGCGGTGTTG TTTTTACGTG GTTATEgtat 17040 agtittitig titigggigit tititigitt tgittigigt tittiggittt áttgittitt 17100 ttggglggat ggggtggtog tagttttatt tigtgttttt tagttgtttt agatttttt-17160 gtittettit teggitting tiattititi attitittat tittititit tegtegTTTT 17220 AGTTITIGATE TTGTGGAAGE ATTTGGCGAT GGATAGATTE AYAATTTGAA TITAGGAGTG 17280 TATATETT ABITETATITE TATETTET ATOTTOPIAN ATTACATOR TITTATETT 17340 AGITTAGITA GOTTTTAGGI AGGGITAATI TITTITTITTI TGTAAATAAA GITTIBGGAT 17400 TATTgtgtgt ogtttttgag ttttttgttt gtttagtgag tgggoggtta gagggtaggg 17460 taggatgggt amtigtgigt gillitogigo gigittogog igasagitto gittittogit 17520 agaoggacut gegiogggat itogittogi ecgigggag gigatogigg gigaagitti 17580 ttagtttttt ttittamaat ggagggogat tataatageg tegttetean angintogag 17640 atgaoggtty mogatangao gegtatagty attiatiate ogtificiat gtgttinggt 17700 attassagat tatataogit agritagitt aggistlitt gitattitta tittatogig 17760 goggagatty aggretages quattorgia attiguttet tigtitagge tietaggett 17820

[図36]

stagastagt gaggitgage ttogasitts gattgitigs tittegagit tatettitt 17880 attitiggagt tgtagtiggg gitattitta ggggggitti gattatatti tiltigatgit gagittings titigantine gangagingt testagiogs associaset tigagging 18000 ttoggttgog ttttttttgg ogggastagg geteretttt ttegggtett ogggteogtt 18060 tagittitit tittatitag giogitgitg titttatitt titggstaga giligasgag 18120 tiggitgacg tgeagagigt tilgillill gillillit tillillita igitegaget 18180 sagattittt tittattiga aatattegig tittgeggang taangtoggt gegagttatt 18240 tittaggang tettegogit tattitigga saggitigaga tagtataggit tittanagitit 18300 egnggttegg ogtgtettet ttegtenett titetegegt titoggogtt steggtettt 18380 stagttitto gattitting satitugges stiggitugg gage 18404

[図37]

ttittttgat tagttittta agittlagga ggtoggagga ttgtgaatgt ttgtgaogit susgetting tengentity tipogenety teogettagt tittgggtit tegentitet 120 stigitting tittitinge agigggogtt agistititi gagggatgat tittatoggi 180 tttatttttt seggtetteg tetttaset sangangane tittettttt getsternung 240 sagguagagg ggatesanss tensetatit titacettag tingititit samititati 300 tengaggata agugtagtag oggittigat gaggggogga gitgggogig tiogagigit 360 tigasgasti istiliisti tiositagas sessostasi osssiissii itagsiitso 420 gittioggit gitsattatt ttitttagit tagattiggs attingtatt aggggngigt 480 gattaggatt tittigaggg tggittlagt tateattita gestangene tutaggitti 540 ggggttagat agtitgggit ogsattitag tittattgit tistagtitt gigattitgg 600 gtengtgatt sagttettta gtttttttgt tttttagttt togttnoggt manatgagan tgatagaagt gittagatte aattemogte tetagtitit tegtetitge etatategta 720 agogigigat gagitatigi gitogittia togitagiog itatitoggi gittittata 780 attattitgt tatgatogtt ittitattita asganggaga tiggggagit itattitacgg 840 thattititt acgigogage oggagittog attimogito gitigmogga aggoggagit 900 titeogogag gtaogtaogg aggletatat agtiatitet titigtitigt tittiggtog 860 tttatttatt gggtaagtaa agggtttaga ggcgatatat AGTGATTITA GGGTTTTATT 1020 TATAAGAGGA GAAGGGTTOG TTTTGTTTGG GGTTTGGTTG GGTTATATAT AGGGTTAGGG 1060 AGAGETOGOG GEGATETAGT TATTTAAATT ATAAAAGAAT GEGGTATTTT TAGGTTTAGG 1140 THETEASTIT CITTATCECO AMATCITITI ATAGOSTIAG COTTGAGGET ELLEAGAGGE skengtynes gagtgagnky gtogitegan titggaggty gantangogy gittggggta 1260 gttgggaagt staggatrag gttgoggtta ttttattist ttagggagg tagtgragtt 1320 saggatatag ggtagggtag ggoggstatt taggtagang agttgtatTA TOSTTATTTS 1280 AGRAJAGTAT CETTTATTIT TITTTEAGGG AATTITTETT TITTITTTE TITGITGATC GITGITTITT TARTITTIT TITTITTET TITTAGTGTG TAGGTTTTTA TATATATTT 1500 TTTTGTGTtt gingtogget ginggreet austateret auttrutata gutterrare 1560 tagtatagge ggattoggta tiggtagttt tttttggtts titogtitta taggtgeogt 1820 egitsoggit titgiating attitusgit tituguggut egyggitete tilitatetec 1880 gagtgogttg tiggtattie giagaggitt atacgtgigg giattiogga anigittate 1740 antaganger gigagesite agittigaett igengetage tittittlegt igianialit 1800 gtttttttta gasagtgagg atogggttgg gtaggatana greetegatg togggtagtg 1860 STORELINER TESTIFICATION OF STREET, SCACOASCIE CONSCIENT ASSESSMENT ATTITITALS 1920 STYGOGGAY ASSTCATORY TICSTATTIC SATTITIONS TITTITIONS Lightening - 1960

[図38]

aggratests aggasstart estitiagte gentlessts stattlettt tetatistes 2040 tttttttttt ttttaggitt igtttigten gietTfGTAS GATITITAST TITITTTTAS 2100 TESTTITAAT GAATTOGGOS ATGGTTACHT AGATGAATTT STATTOCGTT TICSTTTSTA 2160 TTATETTOSA GOSTTOCGIT CSTATTATIT SCATGOTTIT TTOCATETTA AYOTTATAGIT 2220 TTAGGTttax strangate stienstitt sentiteret sterscreet storsgreet 2280 tgittattit ttattittas attittaggt gtittttatT TTTGGTGGAG ATGTTTTTTA 2340 TEAGTATUTC GATGATAATG ATGGTGTTTG TGCGCTCGAT GTCGCCGttg grgangsog 2400 suggigitat tagaggitag tiesgittit ticggenagg sengtattin gogittagit 2460 tingtitteg gististegg agtegitiga gittlingig atgegaggga atgigtiteg 2520 satistogge taugigates tagagettis titagittis tatagatece tittatitics 2580 ggtttigogg tgligaogit ggittittag tigitatiat tittititit titattitto gogtogatti titattitta gigagigatt giittiatti oggaagatti gagattitta 2700 astittatia tigittigio ggittitiagg tattitgagi tittitogii tittitigos 2760 ggagttatgg agogttittt agtatggttt tatttttttt tittittaga tittgaogit 2820 agggaangga tagtitigag atagtagget gogggestat agggitaggg gatitaggg 2880 agtagtgtag taggaagttt ttttttttt ttagtttigg ginnatagat ttingggaga titteggtit attittagti gagtitagta agtigatgtt tigagtogog tigtitastt 3000 tteastitag stigitmans stastititt statitatit tittititate scuttassi 3060 assignitate aggittitus gengining astatetets ingongeres stageseert 3120 agtittoggs gatgagitty tititgagag agtataggat agagtogogt titoggatag 3180 tttatogggg gtattittag attragtoga titgttitgt titeggates stagitgitg 3240 suggrages aggregates aggregatest aggregate thinogens sagings and 3300 3350 ggatoggggt saggtaggit titistititt tatititigt tigatgigag tistisasti egicategog titegittit gegggitgeo ggesttiteg tietesogut egggittist 3420 tgagtitita ogataatitt gagatittga tittatagat gaggangtig aggattagan 3480 sagttangta attigities getteteing tigginagog gaggggittt ginittetts 3540 ttttttettt attiggatta gattagtita atgagtitet tigtgesett tttettttt 3600 titiglegitt tiatititigi attituttag agattititig attatanata igugggtaga 3660 togutogogg agitatetet agittimang magintiatt totttitate togittiess 3720 gettaggatg gestattett tegggaagtt gegagagage gtagagttag gungaagtog 3780 asgittiges ettatogats samansags magnateges agiatatett itatagatet ittiggsaga agaaattaat agaateggat ittogagagaa agongagtas iceggatata 3840 3900 getttattet gegtettet toggttitet titgatingt tiggetitek tiagtaatte

【図39】

ggtttogggt ttttttttg tatttggtag ggttgggogu getggtetat toggagaggg 4020 tittgaggas gatetiteta getegegget tesagtgegg tiggitting timogatita tttogugttt tggagttatg nggggattta ggagaagtog ggggtggggggggtattt 4140 astgigogag timecgitta teligittat astagittat agistitati agistitati 4200 tittegtigt tektettett etteggette tiettitiet TTGTAGTGTA COATGATGGO 4260 TITTECRICA GETAGATITI TITOTOGITO RITEATITGE TITARGAAGT TOAGGATATI 4320 TYTAGGTTTA TTGGGGGATTT TATGGTCGGG TTAGTTTAGG TATTGGTAAT GYTAGATTTT 4380 ICGAATTAGG TITTITtagg torsevatat assettagts tittittitt titograats 4440 tittattiat titattitat togettegag aggagastas gittagggag ggattitogg 4500 matgagging agogtaggag tigttatAT TETTTAGOGG GGAGATITET AAGGTACGGA 4560 OTTIGIATIC GETTETETTA TOTTITTICET ASTIGOTTAT AGAGTAGGET TTATAAGTAC 4620 STIGTATSTT TATTICGGST TAGTATGGGA CSTATTISTT tiggssegg stertogest 4BB0 suggetgage tetagagtag setattetes titatities etitiettit atettiteas 4740 tettigetat oggittaaat attigtigga tegaoggate getteetaga gaggiagag 4800 stitgggtit togttitage tientimite staggiggig egetitggit agitatitie toggtatett ogtitgitat tinatusang tigtiagggt agstatigit tiogsagitg 4920 tutosettet atagtittet agetateest astetttest tigtetitit statticeer ADRO gagittatag agatagatua togggggaga tatogogtte aggagogtes ttessaansa 5040 ogtantitti tittiaggit tiggaggasa taugatting atitotogng gaggtagogt 5100 ttoggtigig tittiassas ogsgetattt gitaggtaga gatagigigg agagagttat ttittegtag manetgagit tgtgtsmaga maggitggga mittgamega gitgengtig 6220 tigggegogg tgagttttta agagangogt goggattaga gagangttat teratitien 5280 stettegett ttagagtgog attituttit anngetgatt aggagttate generagatti **5340** tagenagges gynattigat tingtitgig tinoggaing guttattite atggingini agagggtgtt gtgtaggggg agtttgtagg gtaaggggta gattttagag agatttatgg 5460 entaggrage staggogges titiggatete gateggtese staggogtag garrageme 5520 gatagities sattlegett tegegittes augustites ggatstatet egggetties 5580 ggattaogtt gigtagioga gattitetta tiagittitia gittitiaat sagggiogga ttittattit tgagattitt tittgittig gtattigati gittitoget ttaatggtat 5700 ENTERTIBLE ROGERSONS STRONGERS OF STREETS OF THE STRONGERS OF STREETS OF STRE 5760 5820 ATTENTCATE ETTTTAGAT AATTTTEUTT GOCSATETAG GTTTTAGCOT TITTATTAGO E880 STITAGTAGT TRETTEREN Ogtengogte gagagaogta tittangata gigagttitt

[図40]

testttasta tasetttisa attatistta tititattis tetanganet tastttoset 6000 titetttagt tettittega titagegitt tittattaga tagegaatit ttagegitt gtttttttt ttacgtggtt tatattgttg atTTTGATGT AGTTGGYATT GATGTAGTCG 6120 GATTCEGGGA TETTATTOTT TOSTTITTOT AGGATTATIC GGTTGTGGTT AAttgeggt 6180 EXECUTARES SERVICERES ESTIMANT ASTISSES TRATES DE L'ACTUAL L'ACCUSANT L'ACTUAL SE L'ACCUSANT L' 6240 ttttttgagt tastaggtta tittggtttt agmagttatg aggtttgogs gagatigtgg 6300 gtagtagggg tegeggatta titgtatega gegagtegeg gagtitates gyaggitgeg agtagggggt sattigting thegheries graggistit theggaggag ingaggithi 6420 tisgaguage segassitit signgessig tempgaguts stittitits soutesages 6480 tagagattig igagatgaga saasstagtt gargtagga ogstattitg gatgaatgag 6540 gtegtttggg tgttatAGG GGAGAATGIT TITGTAGCGG TITTTGTTTT TETTTTTTEE 8600 TOGTTOTTET TITAGACCIT GOTGYANGIT TITTATTITT TOTTTTETA AATTELEGE 6660 tetsestess genetages thegittite esgaggitat thatogogy estiteogiy 6720 gesteratte genetarare teretackee generalite thoutities sittlessto 6780 gligitatie tilitagligt titaglittig toggittitia timigiatil TAAATTITTI TTABAAGITA BITTIBBITG TATTITCGBA TITTTBITTI TIGITTABIT TIAATATICB 6900 SITTITAATO TYAGTOSTAT TTATTTCST GGTATAGTAG GGTLEEGELE GEGRASSOGO ROAD agangitagt sattingage titingangt tietatitigg gggatagiti aggagengig 7020 aggaggtagt tgggtttatt tttgatTGT CGTAGGTAGA TAAAGGCGTT TGAGGTTTTT 7080 TTAATTTICO TTITTTIBAA ATRITTTATT AGRITGGGA GGTTGTCGAA GGTTTTTAAA 7140 TTATTTATTO TOTAGCOTTT ATTELEGERS SASTLABALL ASSILLATION INSPECTION gttttttttt tigggttttt agtttignat tinngiting gingnititt titgttatit. 7260 statistizat tatagategy extitizate garettitit ogtoritize tigittistT 7320 TOUTATATOA TITTEATOTO GETEATITTE AGCOGGAAY TEGOTTAGT TITEGETTEE 7380 TTATTGAGTA TAGAAASTAG GAAGTTTTTA GGTTGGTTGA GGTTTTTAGG TATAASAAAC GTTTAGGGTT CGTTTTTOGT TTGTAGTAGT GTTTTTETTT GTTCGTTAGA TATETGGTTA 7500 TEGTATTATE togagangge agogttages saggtaagat ttagettate suggnareog 7560 7620 ttgagtagag atattiatag agagegetog gesattaagt atttattgig tgttaggist tgogttatet gittitaast tiattatiit attinggitt titetesate igitgagagi 7690 taggiatgat attattitta tittatagat gaggamette mogitigagit attattitett 7740 ggittegggt tittgattga tittagggta agtitttage gtittgitgt ttaggtigga gtgfagtage gegettateg tittitgtag tittegatitt ttaggtiten gtiattitag 7800 7860 tittingagi titaningg titogantii agagtitati titganaggi aganaggitg

【図41】

gtantattte mestatting aggagagang thouggaige ottitogiga aggatgagat teigtiting tegngating agglessges searginget ogegetiate tisingggag 8040 tegteggaga tegatatata gatttaugtt stanggagg cegatgeggt gogtttogtt 8100 auguaguoga spaagataat titattouru aaagagtest stassussit tiguagtett 8160 ttggatgatg ttattgmage gengtttage gittemacogs atttgaagge gitgitagge taguanging antiangtit tittitogan ginistitit tittinggan gammitging RORA titiatuang titagitita taggamatta ogigatagna sanntatena stitititia 8340 sugagagagt gagaastgag stigigaggt tittitagtt tagggacgag gegagagagag 8400 stanattitt testogtage gatgegagga aggaggagag gattagtite tittestite ttttetttt gttoggeggt gettitegto gegogettt toggettitt toetttiett 8520 gggttatttt agtttatogt tiggggatag attaggattt taggtagogg ggagggggtt 8580 gogttttttt tittaggttt toggatgage augttittat etaestetta getteattet 8640 aggaegogta gagttatoga gggutagatg taaattttog tagaggagga ggogtataga 8700 tonggtagag gatatagtag muttigggag anngagtitit tietoiniti tinggiting aggigetett agtittiggs acggettatt tessaggett tittagtgogs tittitiggt 8870 tttaattttt ogtttoggta tigtitittt ttttttttt ttttttttt titttttta 8880 atttaggitt tagttttitt tgtaggggtt tittggggtt ttgtttttt tittlatitt 8940 taggtattee ttagttogtt ttitttttt egttegaget noggogonng ngaggatatg 9000 ngittingng metitoggig tiansangtit titgngigtt goggiggage agaggangag 9080 tagggggtag stanggagtt agitatgige getttatuge gegagggeng gittigsagg 9120 sgrtogettt aggenogges gittittagg ignettetag gettetatog ignicenati 9180 tagtitians titlesting tettinett assautaits sigtagiting sagettomas 9240 ttttagagag ettetaagit getoggagta agyttttoga ttatitiaat asiogattia 9300 tgantgtagg gutttacggt augggtaugt cggtttggtt tuggggtgtu tgtganaatt 9380 ttttagogtt atttogtigt tttttatttt angagggettt getettgaga tecepttett 9420 granggraft atggigttet gaggagtatt tagagttttt agittitagt ttatgttttt 9480 segngattat aggaggagto ggggoggtta tagganggna atgittggag tattattitg tettogerat ogogtgagat attitatata getgaatati tiotatogig tittiaatga 9600 tttttettix gatoggagat ttatttgatt ttataggata gatttatttt tacaggagga 9660 tigggates glatiougig titiggies tettiatit toititiatt tragigites 9720 titittatte tittaatate tettereete aggestagat gearganget aggitogati ttagaggaag magagttttt tantastaga gttggttigt mogatatgtt gettattagt 9840 agtestitt tigtitting teggattige gitgiagoge tiaggettit oggastages 9900

【図42】

ogggatestt tittetestt tittosgest tegetesese eggtestese tierettite 9960 agotattaga tittaggata stagggagga segatattea gagtittagt tegtetagge 10020 aggagaggag atgaaggeta sytagtette ettettagit tittigitti titigettett 10080 tagustessa storettiat tittigittes saatesoges estsogeast tittatiiii 10140 TATTAUTOGG ATCGCARTAG TITACCGCGT ATTTGAGGTG GATGATGCTG TCGTCGCGGT 10200 TITETAGOAT ATTITETET TOAGTETAGT ATTITATIAG TITTETTAGA GTOGTAAAIT 10260 TITITITITE ATATAGGITA TAGAAATTIT TIGAGTITIG GATICGAATA TGGGTTATIT 10320 CATITITAT Tetgtagggt attaggoggt gaggttagto ggtgtaggog taguggtagg aggstagesa toggttgagg tittigggtgg gggtatatat gggtatgggt gggtatagat 10440 aggratures agisteress tiegrastes aggrataget exercettes tetigitees 10500 augusttage autgagtigt ttagggittig gatggtates gagugtggit susstigting 10560 SERVICERE SERVICENTE STATEMENT STATE GEGITTOAGIT AGGAAGITAT COTGOATATT TCGGTTTTTG AGTAGGGTTT TTGTATTTAG 10680 TITATIGAGG TITCGGTGAA ATTATITERE CERTITERET RESETARIER FRANKERETET 10740 cetegetett aggistlitt attititett tatoeteget estaggitt tatTTTATTA 10800 TTTT00000T TTTC5GAGAG BAADO85GCB TAGSBAATEA OQAOCTOTAG TTAETTT5556 10860 TAGGTAGAGA GITTAGGGAT TAAGITITAG ATGTAGITIT TAGTGTCGGG TCGTTTTATT 10920 ATTITOGETO GITTIAGITI IGGGGTTGIT ATTITATTEE tttggggtag toggtagggc 10000 ERRESTERRE ERREGORGER togettittt provinstit attigtatit tiogtities 11040 aptatagten gggengagen gtagngtage etgetatter regtagegeno gjegtttatt 11100 tittataogt tiginatten aggittagga gtatggiett gistatetetgi gainetatet 11180 gingineges tatatgigig.tttigiatet titiaggilia getentattt tacginigeg. ..11220 satitagang gittitanggi attitititi tititating tigititiso giagtititi 11280 tiggitiacy tinetnetes testagoget estastigit storagions tiligitums 11340 tgtogggogt tgtgttataa tggogtttat atgttttgtt tgottggitt tttatiotag 11400 ttttatgama taggggitat tittattatt ttatagatam gganttigam mittagggam 11460 tittangatat tetitgagit tattagtgag tiagtigtag tettaggatt tanatitaga 11520 tagttogett tiessettes tetttisest tittetetet tagtitities setttieses 11580 antogogati negagititt tigittaneg gugitgitet gggngiagga gattasiagi 11640 teagetttty timetetty teagegages gggtgttttt tittestice teletytest tigittatet tatigiggie gegggatgge testatgett tigagiestt tigitesini 11780 aggittitti testittist ittisagitti tigittigit tiagigitti tattagger 11820 titatititit gggggtiggg sgataaggit titattetgg tigattitit tegggtiggt 11880

[図43]

tgattitaga ggttttasat attasggtat etgatgttgt ttattigtas tittasatat 11940 esatetteat atogitzeat tesanogage managiagit tentegeren intelagean 12000 statatatte stystiitit tingsgares naatteeste stiggaggat aggetteese 12060 gogotottig giittitata tattititig aatgitatat gaigggtate tattattitt 12120 stangates stateticat texpestest titterroom tititegest stareroome 12180 ttttttgitt ttmittggat gitgittgte teggigtgte tensogigse gitggtoggs 12240 ogtagteett taogitteta attitagtat titregesent tasastisso seattacear 12300 gttaggagat ogagattatt tiggitaata tegigaanit tittittiot taanaatata 12360 sessesting tiggetates testaggoet tigtestitt estiations gengitemag 12420 taggagastt attigastit aggangtoga gettgtagug agttangatt atattettet 12480 attituatit primatatar tangatitta tittunouna nonconnuna teraptters 12540 tigigigitit maintitiate maintitatet atgittigit tieggiomen gragmannen ttttaugtit autaugtite attttttat gataatista tiettetitt tagaaatatt 12660 assistiast stittittt assassarett tilltelitt tilttettt ttettetti 12720 attigittit ggittitti tittatiggt gittigggga attigittgg tgrgittati 12780 giatggitta gittigatta tittittagi tiggataita tittegagit toggasetto 12840 gogganttit taogittaso gitaspragi sagaagagta taigiasagi tagitatago 12900 gogggotatt ogtiggogit tiogittatt toggagigtt tagagittag tecgatetti 12960 ggtattgagt sagtgtitas tanatnagtt cattagtita tagttatata titggttata 13020 agintegtti titggittin tatatetagi tigtitgita taogteggia egggigiata 13080 tatagitista giatatatat tittettatit atgittatit atatatatat tiggitoggi gistatititt acgistggss tatesettig stistatog segittings titatgtits 13200 stationace titettetat titestitita againtititi titeseetti santitatti 13260 ggtgggaagg aattaattog gtattieges ganatiestt aggtattitt tittgstite 13320 tegengaget appraisitt tignappete tiongitung agetageatt togetageng gtatagttit tgttattitt tggtaggasa etassanett attittttta ttttggtatg 13440 stategesett tetagattat tittetatat accentitiat itegetitia satastitis 13500 tgatgtagga attattitti ttattitti sigggoggaa staagogtag ggagsitang 13560 astiniam giasatgiti gainstiing magaansaan tettitiin ganginggon 13620 gotnessgie gegetteegg tteggogteg tggitteogt tigteettit egtettitgi secretaria tigriguati attigatati aagtitunga stastitung santitusas 13740 assittigit titutossas statement testimorio instastate tittigiasi 13800 titagetatt tergagatig agategrage atogittene titerestes togagettet

【図44】

agtgagttat ggtigtgtts tigtasttog gttigggtga tagagigaga ttitgttita 13920 assenteens stategitgy gogtegigst tistatitgt satitiogia titigggage 13980 togaggtegg aggattatit gaggttagga gttogagatt agitgggtas tetagtegan 14040 ttttattitt attagtitgt antittagtt tittaggagg tggaggttgt agtgagtoga 14100 gattatgita togtattita gittgggtun tagugtung titogittin ananananan 14160 antissessi tatatagatt sagattante signistance sagatagata stiturango 14220 gisenstange tematestt igingenngi titggentig tittgegitt ingestitto 14280 gttigatean tgagttient anintittit mentattgat gtgannagtt gginngtest agggilling igitgitagi tittatatta taltattaat tittaggita attitatita 14400 giaggigita titattisat titagasata geneticasa titagasata tinaginati 14460 gittaugutt ataagttagt gagtaggaag gtgggtanna tanggttgtt agangtngga 14520 gttiggtett tttttttta atastistat tgtagtagat agogtoggge gtagategga tegetattet tataettiat enternaget generaagen etettittit agaegianie 14840 tegettegge temagaggig giacottotag agittatate ggtogtgttt agitogtgagi 14700 angitatety ettreasion ogitatitis agareteese attitutist agasestat 14760 tteaggitag gitgagasat teaggatgas attigietta gyggaggitt ggitaagita 14820 titiggiagen annigatiot aganggratit agggatiaga agatitogia gynatitiat 14880 orriganeto entagtenta agatetetat terretagre etagataten etetpapuar 14940 teagesttag taggettage gauttettta ittateamit cettagtett tigtaggtag 15000 gtigitatit tittititit tittititit tittigagat geagtititt togifetati . 15080 taggitggag tgtagtgaog tastttoggt ttattgtaat tttogttttt tgggtttaag 15120 testititit gittingtit tillagtagt teggesthat negtgogtat tettgigtha 15180 gittaatitt tilltigist titlagtiga gatagggitt tattatgtig gitaggligg 15740 tittgaatit tigatitogi gattiattig tittegattit tiasaatatt aggestatag 15300 gittgagita ttatgittag toggitgita ttittitti tillitioga toggitgita 15380 terretter terretter terrettes attracture attractive attractive 15420 THISTITE CONTROL THISTITE CONTROL TO SECURITE STREET 15480 TETETETE TETETETE TETETETET TETETETET TETETETET TETETETET 16540 tttttttgat atagasittt gittistist tinggitgas gigtagigit singiliant 15600 ttettgtego glogatttit tiggttiagg tgatttittt gttttagttt tttmagtast 15660 tragattata getgtatett attatattia ettattiatt agtagtaata stagtogtag 16720 togteginge gatgaggitt tittttatgt tgiltagggt ggittigaat tittigggttt 157B0 ongignitti titattitag tittitaani igiinggati nitatatgit iggattatin 15840

【図45】

tatitagtat tittittiaa tagagutggg gittititat gitgittiag tiggittiaa 15900 stittigagi tiangtasti tittititto ggittittas agigitgaga tiatagaegi gogitattit attiogitag gittitatti tisatattit agittiaata iggittasti 15960 16020 igligiggit tigilitogi titattaggg tittitigita ogggiatati gtittititi 16080 tgtligtttt tatattaggg tttigtgtit attatitttt ttgtttagag etgtliggit titigattati acgitatatg gitagittit tittittitt attogggitt tagittamat 18200 gitattittt oggganggit tiltigatti tittagitag fantgitatt igistattin 18260 gitetitett agiatatgan gitggittat tittititt tittilitt itgagataeg 16320 gttttatttt gtogtttagg tiggigtgta giggigtaat tatogtigat igingittig attititage titaagtaag tittitatit tagtititit stagtiggan tintaggang 18440 tgtgttatta tatttagtta attititatt tittgtagag stegggittt attatetteg 18500 ttaggitggt titamattit igggittang tantititit gittingtit titatagigt 16560 typesttata gytytysatt attatettog gtimagetty tttatttttt goginatett 16620 tttgagsatt tgisatgatt tenttiattg ggitattigt toggittgia attitttatt 16880 ggaatgtang titogtyago gtagagatta tgtttgttit gtitatitta gtattattag 16740 tettitasat agtattiggt giataataga tettitastac stattigits satgastest 16800 gteggggen gggnegtyen eggmentenn gangatgggn thanngtity styttgggan 16860 gigoggagna gitggitgga ogigggaggg tittlogoggg iggittitga itgitatgit 16920 transtatte transtatt aggregates stittaagta grasstages staggagtit 16980 agenetteen agtogonate testegages antagateta tettigitta suggamente 17040 thouseness agendades tatgagedes towassett thatetitte offensess 17100 sagittatas gratograsa taggogtase stagogetas atogtatita ogittatata 17160. ogtoggagtg agtattanta aagttttttt titttitama sitaatagga gattaggatt toggatggtgt togggattta tytacgogat AGTATTTTT TGTAGGAGG GGGAGATTAC 17220 17780 GOCGOAGOST CSTAGGGGGC GOSGTTGAST TAGCOSTATA GITGTGTATA GIBSTITAAS 17340 TRADITION ASSAUTCH THE TANTANT TATTETT TECONTITUE THE TECONOMICS TO THE TENTANCE TO THE TENTAL TRADITION OF THE TENTAL TRADITI 17400 TAATgetatt gingggaten ettitiggett antegtittt aggnanegge tegetgegge 17460 surgitigati operatogit totagoggit agtititite tegtitiese gogggagtet 17520 satesattes titatiggit taggetogit tittitegga egeggeggeg tiatitiggar 17580 ttttatttgg agtttaggtt gatagagogg ogggstattg ggtaggugan tegettagga 17640 agiantitis gitengrang agiantical gaugement tittogiage ogganatica 17700 aggettteng generatittt nategitteg tillttagag naggaggiet tittletate 17760 sgittattta tttagggogt tgagggatti attitagtti tttaattigi ttogggggam 17820

[図47]

(a) Wild type DNA

5' -AGCTCGCGATGCCAGCTCGCTCG-3' sense strand
3' -TCGAGCGCTACGGTCGAGCGAGC-5' antisense strand

(b)

Bisuilited

5' -AGITCGCGATGTTAGTTCGTTCG-3' sense strand
3' -TTGAGCGCTATGGTTGAGCGAGC-5' antisence strand

(c)

FW primer 5' -AGTTCGCGA

5' -AGTTCGCGATGTTAGTTCGTTCG-3' sense strand
3' -TCAAGCAAGC-5' RV primer

3 -10AAGGAAGG-0

(d)

FW primer 5' -AACTC6CGA

3'-TTGAGCGCTATGG<u>TTGAGCGAGC-5'</u> antisense stran 3'-<u>TTGAGCGAGC</u>-5' RV primer 【図46】

sessesset titagetass stiegettit gegetagest togetitast tagentiate 17880 assessed attraces eterestic generators resentiare reagrapses 17840 tgggggttgt taggttogts agagsttgat atteaggtts agitateggo gtatttatte tigitigges tatleaggit tittititit iggiegiggt staegggegg gitzettiti 12080 aggaggoggt tacgtigtts tiagtogtag gittateggg tegtagggtt sigggoggta 18120 generategogt tittegtite titteateget tegeogetitt aggettitge gittogtage 18180 attigation gogagioent tienggatet tigiongegg gagtggengg tienttittt 18240 ttttmagget tegogtgitt segsteitig geggggsigg tegittmagt atgantigit 18300 tttttattes tterstegit gittgitess teggeogist gteggittit gittogists 18360 stitusates testatites titatettes statesting ates

[図48]

(m)

REP-S1: 5' -CAGGCCAGTGGAGTGGCAG-3'

(b) ... REP-AS1: 5'-GAGGAGGTGCAGCTAGTCTG-3'

(c) (#7441)

CAGGOCAGTGGAGTGGCAGCCCCAGAACTGGGACCACCGGGGGTGGTGA
REP-81 Hoall

GGGGGCCGGGACTGGGAGCTGCATCTGAGGCTTAGTCCCTGAGCTCTCT Hpall

GCCTGCCCAGACTAGCTGCACCTCCTC (#7566)
REP AS1

【図49】

(**a**)

REP-S2: 5'-CAAAGCACTGGCTTTGGAACC-3'

(b)

REP-AS2: 5'-ATCGAGTGAGTCCTGCTGGAT-3'

(c) (#6858)

<u>CAAAGCACTGGCTTTGGAACC</u>GGACTGTCTGGGTTTGAATCCTGGCACTG REP-62 Hpa II

CAGCTGACTGATGGACTCAGGCAATGCCTTAAACTCCCTGAGCCTC

AGGTTCCTTGTCTGTAAAATGATAAAGATAGCCCCTGTTTCATAGGGCTGT

GGTGAGAAACCAATCABACAAGGCATGTGAACGCCATTATAGCACAGCG

CCCGGCATCCAGCAGGACTCACTCGAT (#7084) Hps II REP-A82

•

[図50]

SHP1-PF1: 5'-TGTCTGGAGGCCACGGTCAATGA-3'

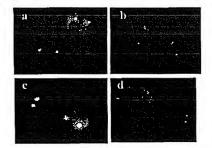
SHP1-PR1: 5' -GTTTGTATTCGGTTGTGTCATGCTC-3'

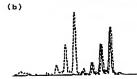
[図53]

(a)

FISH analysis of ILTMot and NK-YS cells with chromosome

cells	positive signal No.(%)						
	probe	0	1	2	3	4	more
ILTMot	Сь#12	1	1	97	1	G	0
	SHP1	1	2	95	1	1	0
NK-YS	Ch #12	0	0	99	1	0	0
	SHP1	1	3	91	4	1	0





Microsatellite marker	LOH			
D12S356	15/19 (79 %)			
D12S336	6/16 (38 %)			

【図51】

(a)

SHP-LF1: 5' -CCCAGTTCATTGAAACCACT-3'

SHP-LR1: 5'-CCTTGCTCTTCTCCTTGTCT-3'

【図52】

(a)

MF2: 5'-GAACGTTATTATAGTATAGCGTTC-3'

MR2: 5'-TCACGCATACGAACCCAAACG-3'

(0) (#7037)

<u>GAACGCCATTATAGCACAGCGCCC</u>GGCATCCAGCAGGACTCACTCGAT MF2

GCSTAAAAGCAGCTBGTGGAGGAGGGABAGATGCCGTGGGAC<u>CGTCT</u>

GGGTTCGCATGCGTGA (#7195)

フロントページの続き

F 夕一ム(参考) 4B063 QA01 QA13 QQ08 QQ33 QQ43 QQ53 QQ62 QR08 QR14 QR32 QR50 QR50 QR62 QS11 QS16 QS25 QS34